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13. ABSTRACT (Maximum 200 Words) This is the Final report on the grant "Proto-oncogene PML and tumor evasion in prostate cancer". We proposed to identify the antigen presentation defects in human prostate cancer samples and to use mouse prostate cancer model (TRAMP mice) to study the immune regulation and immune tolerance in prostate cancer. In the past three years, we have completed the experiments proposed in the grant. We have shown that proto-oncogene PML and the major histocompatibility antigen HLA class I are concordantly down-regulated in high grade prostate cancer. Using mouse prostate cancer model, we have shown that thymic clonal deletion is a major mechanism for immune tolerance to tumor antigens that previously regarded as prostate specific. We provided the direct evidence that the T cell repertoire specific for tumor antigens can be shaped by negative selection in the thymus. We identified a new novel mechanism for antigen presentation gene regulation, i.e. the degradation of mRNA of an antigen presentation gene was involved in tumor evasion of immune recognition. We analyzed the transcription regulation of one of the antigen presentation genes and identified two new promoter regions and the essential role of the interferon response factor-binding element (IRFE) in that promoter region. Finally, our preliminary data from chimera mice suggested that distinct thymic cell types expressing peripheral tumor antigen have different roles in determining the range and degree of central and peripheral tolerance.				
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(4) Introduction

The peptides presented by class I major histocompatibility complex [MHC, in human, human leukocyte antigen (HLA)] are the primary targets on tumor cells for immune recognition by host cytotoxic T lymphocytes (CTL). Tumors can therefore avoid CTL recognition by down-regulation of cell surface MHC class I molecules. We have proposed to investigate the molecular mechanism of tumor evasion of host anti-tumor immunity. First we will identify the antigen presentation defects in prostate cancer. We will examine the role of proto-oncogene PML in HLA class I down regulation in prostate cancer. We will determine whether PML malfunction is responsible for HLA down regulation in prostate cancer samples. Furthermore, we will examine the immune regulation and tumor evasion mechanisms in experimental transgenic murine prostate cancer (TRAMP) models. During the first funding period, we have performed immunohistochemical study to show the concordant proto-oncogene PML and HLA class I antigen down-regulation in surgically removed prostate cancer lesions. We have examined the proto-oncogene PML isoform expression and antigen presentation gene expression in prostate cancer cell lines. In second funding period, we have shown that thymic clonal deletion is a major mechanism for immune tolerance to tumor antigens that previously regarded as prostate specific. We provided the direct evidence that the T cell repertoire specific for tumor antigens can be shaped by negative selection in the thymus. During the process of examine antigen presentation defects in different tumor cell lines, we identified a new novel mechanism for antigen presentation gene regulation, i.e. the degradation of mRNA of an antigen presentation gene was involved in tumor evasion of immune recognition. We have also analyzed the transcription regulation of one of the antigen presentation genes and identified two new promoter regions and the essential role of the interferon response factor-binding element (IRFE) in that promoter region. During the third funding period, we have launched a new project to examine the hypothesis that rescuing high avidity T cells from negative selection by blocking the costimulatory signals may provide the innovative immunotherapy for prostate cancer. We have generated chimera mice to study the role of thymic epithelial cells and bone marrow derived dendritic cells in thymic clonal deletion and peripheral deletion. We have generated solid preliminary data to secure a new round of "Idea Development Award" from the U.S. Army Medical Research and Materiel Command Prostate Cancer Research Program.

(5) Body of Annual Report

Task 1. To identify the antigen presentation defects in prostate cancer.

- *Develop the patients' database for 308 cases of radical prostatectomy specimens we collected during the past four years. (In progress).*

Under the support of this grant and a grant from the Cancer Research Institute, we made significant progress in establishing a new prostate cancer tissue resource in The Department of Pathology at the Ohio State University. We have a research nurse, Ms. Robinson, to coordinate the patients' consent to donate tissue for research purpose. She also started to work on the prostate cancer database. We currently collected about 120 fresh frozen samples from patients underwent radical prostatectomy. We also started to utilize the formalin fixed paraffin embedded tissue to produce Tissue Microarray. Each microarray slide contains 140 individual cylinders of tissue of 1.0 mm, which is much larger than other currently available tissue microarray, to provide more information from each tissue section.

- *Identify the prostate cancer samples that have complete loss of HLA class I expression by performing immunohistochemistry study on the archived formalin fixed paraffin embedded prostate cancer tissue samples with anti-HLA class I antibody HC10. (Completed).*

We have completed this task. The work was presented at Experimental Biology 2001, at Orlando, Florida, on March 31 – April 4, 2001. The result was summarized in paper entitled "Concordant down-regulation in expression of proto-oncogene PML and the major histocompatibility antigen HLA class I in high grade prostate cancer". The manuscript is submitted to *Cancer Immunity*. It is attached as Appendix 1.

- *Collect the HLA class I positive tumor tissue, HLA class I negative tumor tissue, HLA class I positive normal or hyperplastic prostatic tissue by Laser Capture Microdissection (LCM) on ethanol fixed paraffin embedded tissue sections. (In progress).*
- *Systematically examine the mRNA expression of multiple genes devoted to MHC class I antigen presentation, including MHC class I heavy chain, β_2M , TAP-1, TAP-2, LMP-2 and LMP-7 by RT-PCR and real time PCR among different groups of tissue. (In progress).*

Task 2. To examine the role of proto-oncogene PML in HLA class I down regulation in prostate cancer.

- *Examine the PML protein expression in prostate cancer by immunohistochemistry study using monoclonal antibody against PML (PG-M3). (Completed).*

We have completed this task. The work was presented at Experimental Biology 2001, at Orlando, Florida, on March 31 – April 4, 2001. The abstract is attached as appendix 1.

The manuscript is in preparation.

- *Examine the correlation between down-regulation of HLA class I and expression of PML protein by examine the mRNA expression of PML from the tissues being collected by Laser Capture Microdissection (LCM). (In progress).*
- *Purify the genome DNA from ethanol fixed paraffin embedded tissue section and sequence the PML gene to determine if PML mutation(s) may be responsible for the defective expression of cell surface HLA class I. (In progress).*

We have systematically examined the HLA class I and PML isoform expression in various human tumor cell lines, including prostate cancer cell lines (Du145, PC3, LnCap), melanoma cell lines (SK-Mel-19, 1092, 1195), and small cell lung carcinoma cell lines (H146,

H1095). We performed Northern Blot experiments to examine the mRNA level for antigen presentation genes, including MHC class I heavy chain, β_2M , TAP-1, TAP-2, LMP-2 and LMP-7, as well as PML isoforms. During the process, we found that in a melanoma cell line SK-Mel-19, the MHC class I expression was severely depressed and transfection of PML cDNA did not restore the MHC class I expression, which was different than what we observed in a murine plasmacytoma cell line. Further analysis of this cell line revealed a novel mechanism for tumor cells down-regulate MHC class I expression. This work is summarized in Appendix 2: "A single nucleotide deletion leads to premature termination codons and degradation of TAP-1 mRNA: a potential novel mechanism for tumor evasion of host immunity". The manuscript is currently under revision for *J. Biol. Chem.*

In related question on transcriptional regulation of antigen presentation genes, we have carried out a detailed study on transcriptional regulation of TAP-2 gene. The work is summarized in Appendix 3: "Cis-elements for TAP-2 transcription: two new promoters and an essential role of the IRFE in interferon-gamma-mediated activation of the transcription initiator". The manuscript has been published in *International Immunology*. This is attached as appendix 3.

Task 3. To test whether overexpression of PML and upregulate MHC class I expression in vivo will improve the overall prognosis in experimental murine prostate cancer model.

- *Obtaining "TRAMP" mice from The Jackson Laboratory. (Completed).*
- *Examine the cell surface MHC class I expression in prostate cancer samples from TRAMP mice to determine whether down regulation of MHC class I heavy chain, β_2 -microglobulin (β_2M), TAP-1, TAP-2, LMP-2 and LMP-7 correlates with progression of murine prostate cancer. (Superseded).*

We have examined more than 50 samples of prostate cancer that developed in TRAMP mice. We did not find any down regulation of MHC class I expression in prostate cancer. In accordantly, we did not perform the work proposed in this subtask.

- *Breed TRAMP mice with β_2M $-/-$ mice to determine whether ablation of MHC class I will accelerate tumor progression (Superseded).*

Since we did not observe any difference in MHC class I expression on prostate cancer in TRAMP mice, suggesting the MHC class I expression may not play any role in tumor progression in this mouse tumor model. We did not perform the work.

- *Make the PML transgenic construct under the control of rat probasin promoter which has tissue specific expression in prostate gland (Completed)*
- *Produce the PML transgenic mice that over-express PML in prostate gland. (Completed).*
- *Breed the PML transgenic mice with TRAMP mice and determine the effect of PML on antigen presentation and tumor progression. (Completed).*

Although we made the PML transgenic mice and PML/TRAMP double transgenic mice, we failed to identify any effect of over-expression of PML in prostate gland on MHC class I expression level, neither we find any effect on tumor incidence or progression. We considered the reason behind the failure was the prostate cancer in TRAMP mice did not down-regulate MHC class I expression.

- *Produce the costimulatory molecule B7-1 transgenic mice under the control of rat probasin promoter. (Completed).*
- *Breed the B7-1 transgenic mice with TRAMP mice, and furthermore, breed the B7-1, PML transgenic mice with TRAMP mice to examine the effects of B7 or B7 plus MHC class I expression in vivo on the incidence of spontaneous prostate cancer (Completed).*

Although we made the B7 transgenic mice and B7/TRAMP double transgenic mice, we failed to identify any effect of over-expression of B7 costimulatory molecule in prostate gland on tumor incidence or progression. We considered the reason behind the failure was that T cells that reactive to tumor antigen (in this model, tumor antigen is SV40 large T antigen) were deleted in thymic development as we reported below.

The transgenic adenocarcinoma of mouse prostate (TRAMP) model is transgenic for the SV40 large T antigen (Tag) under the control of the rat probasin regulatory elements. The TRAMP mice develop tumors spontaneously and orthotopically with a disease progression that closely resembles the progression of human prostate cancer. Previous studies showed that T lymphocytes from TRAMP mice are immune tolerant to SV40 Tag, while the mechanism of the tolerance is not clear. We have demonstrated that clonal deletion is a major mechanism for tolerance to tumor antigens that previously regarded as prostate specific. This work is summarized in Appendix 4: "Clonal deletion of SV40 large T antigen-specific T cells in the TRAMP mice: an important role for negative selection in shaping the repertoire of T cells specific for antigens over-expressed in solid tumor". The manuscript is published in *J. Immunol.*

On the third funding period, we continue to build the Prostate Cancer Tissue Resource with the funding from this grant and from the grant from The Cancer Research Institute. We have continued on work in Task One and Two on Laser Captured Microdissection on different prostate tissue to identify the molecular mechanisms of tumor evasion in prostate cancer.

We have launched a new project based on our recent finding in TRAMP mice that clonal deletion is the major mechanism for immune tolerance. Recent studies have shown that many peripheral antigens are expressed in specialized cells in the thymus and can induce central tolerance. It has been suggested that thymic medullary epithelial cells are responsible in tolerizing developing thymocytes, while some studies pointed to the bone marrow (BM) derived dendritic cells (DC) and macrophages. We have recently taken a double-transgenic approach to show that clonal deletion plays an important role in immune tolerance to tumor antigen SV40 large T antigen (Tag) in prostate cancer mouse model TRAMP mice. Our preliminary data suggested that both BM derived APC and thymic epithelial cells express Tag in the thymus. We carried out a BM radiation chimera mice study to determine the contribution of BM derived APC vs. thymic epithelial cells in clonal deletion process. We followed the fate of Tag specific transgenic CD8⁺V β 8⁺ T cells in thymus and spleen from different BM chimera mice that have distinct cell type expressing Tag. The results showed that clonal deletion of Tag reactive transgenic T cells is induced in the mice that expressed Tag by thymic epithelial cells alone. The clonal deletion is aggravated in mice with both thymic epithelial cells and BM derived APC expressing Tag. An interesting observation is that the Tag specific T cell numbers, as well as antigen specific T cell function, are significantly decreased in the spleen from the mice that Tag is expressed in BM derived APC alone, although such T cell numbers in the thymus are similar to that of the control mice without clonal deletion. Our results suggested that distinct thymic cell types expressing peripheral tumor antigen have different roles in determining the range and degree of central and peripheral tolerance. The result is presented at the 31st Autumn Immunology Conference on November 23 - 25, 2002 at Chicago, Illinois. It is attached as Appendix 5.

(6) Key Research Accomplishments**Year 1:**

- We have examined the concordant expression of proto-oncogene product PML and of HLA class I antigens in 37 surgically removed prostate carcinoma lesions.
- We have systematically examined the HLA class I and PML isoform expression in various human tumor cell lines, including prostate cancer cell lines. We performed Northern Blot experiments to examine the mRNA level for antigen presentation genes, including MHC class I heavy chain, β_2M , TAP-1, TAP-2, LMP-2 and LMP-7, as well as PML isoforms.
- We obtained the TRAMP mice as scheduled and established our own TRAMP mice colony in our animal facility.
- We immunized the TRAMP mice with an immunodominant SV40 Tag epitope IV (peptide 404-411) that is presented by MHC class I molecule H-2 K^b and analyzed the antigen specific T cell response by ELISPOT. We confirmed the previous observation that T cells are immune tolerant in TRAMP mice.
- We have obtained a TCR transgenic mouse model TG-B mice that is transgenic for a rearranged T-cell receptor that recognizes Tag (peptide 559-576) presented by the class I major histocompatibility complex molecule H-2K^k.
- We have further mapped the SV40 Tag epitope to peptide 560-568. The transgenic T cells from TG-B mice respond vigorously to this nonpeptide both in proliferation assay and in cytotoxic killing assay.
- We have crossed the TRAMP mice with TG-B mice. The analysis showed complete thymic deletion of SV40 Tag reactive T cells occurred in the double transgenic mice.
- We have shown that SV40 Tag mRNA can be detected in thymus and spleen by RT-PCR and ECL Southern Blot in TRAMP mice and in TRAMP/TG-B double transgenic mice.
- Our study showed that thymic deletion of T cells specific for SV40 Tag is the major mechanism for T cell tolerance in TRAMP mice.
- We have produced the transgenic mouse that has proto-oncogene PML under the control of rat probasin promoter, and the transgenic mouse that has co-stimulatory molecule B7-1 under the control of rat probasin promoter.

Year 2:

- Under the support of this grant and a grant from the Cancer Research Institute, we made significant progress in establishing a new prostate cancer tissue resource in The Department of Pathology at the Ohio State University.
- We started to utilize the formalin fixed paraffin embedded tissue to produce Tissue Microarray. Each microarray slide contains 140 individual cylinders of tissue of 1.0 mm, which is much larger than other currently available tissue microarray (such as the microarray slides from NCI and U Penn), to provide more information from each tissue section.
- We have shown that thymic clonal deletion is a major mechanism for immune tolerance to tumor antigens that previously regarded as prostate specific. We provided the direct evidence that the T cell repertoire specific for tumor antigens can be shaped by negative selection in the thymus.
- We identified a new novel mechanism for antigen presentation gene regulation, i.e. the degradation of mRNA of an antigen presentation gene was involved in tumor evasion of immune recognition.

- We have analyzed the transcription regulation of one of the antigen presentation genes and identified two new promoter regions and the essential role of the interferon response factor-binding element (IRFE) in that promoter region.

Year 3:

- We have shown that in mouse prostate cancer model TRAMP mice, both bone marrow derived antigen presenting cells and thymic epithelial cells express SV40 large T antigen in the thymus.
- We have carried out a BM radiation chimera mice study to determine the contribution of BM derived APC vs. thymic epithelial cells in clonal deletion process. We followed the fate of Tag specific transgenic $CD8^+V\beta 8^+$ T cells in thymus and spleen from different BM chimera mice that have distinct cell type expressing Tag.
- We have shown that clonal deletion of Tag reactive transgenic T cells is induced in the mice that expressed Tag by thymic epithelial cells alone. The clonal deletion is aggravated in mice with both thymic epithelial cells and BM derived APC expressing Tag.
- We have made an interesting observation that the Tag specific T cell numbers, as well as antigen specific T cell function, are significantly decreased in the spleen from the mice that Tag is expressed in BM derived APC alone, although such T cell numbers in the thymus are similar to that of the control mice without clonal deletion. Our results suggested that distinct thymic cell types expressing peripheral tumor antigen have different roles in determining the range and degree of central and peripheral tolerance.
- We have submitted a new proposal to MRMC PCRP for "Idea Development Award" based on our work in this funding period. The grant proposal (PC020104) was received with high enthusiasm and was recommended for funding.

(7) Reportable Outcomes:**Publications from 1999 to 2002:**

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2. **Pan Zheng**, Supria Sarma, Yong Guo, and Yang Liu. 1999. Two mechanisms for tumor evasion of pre-existing cytotoxic T cell responses: lessons from recurrent tumors. *Cancer Res*. 59:3461-3467.
3. Xue-Feng Bai, Jin-Qing Liu, Xingluo Liu, Yong Guo, Karen Cox, Jin Wen, **Pan Zheng** and Yang Liu. 2000. The heat-stable antigen determines pathogenicity of self-reactive T cells in experimental autoimmune encephalomyelitis. *J. Clin. Invest*. 105: 1227-1232.
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13. Xincheng Zheng, Jian-Xin Gao, Huiming Zhang, Terrence Geiger, Yang Liu and **Pan Zheng**. 2002. Clonal deletion of SV40 large T antigen-specific T cells in the TRAMP mice: an important role for negative selection in shaping the repertoire of T cells specific for antigens over-expressed in solid tumors. *J. Immunol.* 169: 4761-4769.

Manuscripts submitted:

14. Xue-Feng Bai, Jinqing Liu, Xinglou Liu, **Pan Zheng** and Yang Liu, 2002. Transgenic T cell-therapy of large tumors select for antigenic variants. *Submitted to Nature Immunology*.
15. Jian-Xin Gao, Jing Wen, Michael A. Caligiuri, **Pan Zheng** and Yang Liu. 2002. Two-signal requirement for activation and effector function of natural killer cells in tumor rejection. *Immunity (revision under review)*.
16. Tianyu Yang, Beth McNally, Soldano Ferrone, Yang Liu and **Pan Zheng**. 2002. A single nucleotide deletion leads to premature termination codons and degradation of TAP-1 mRNA: a potential mechanism for tumor evasion of host immunity. *JBC (under revision)*.
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3. Yang TY, McNally B, Ferrone S, Prior TW, Liu Y, **Zheng P**, HLA class I antigen presentation machinery defects in the melanoma cell line SK-MEL-19. FASEB 2001; 15(5):A1180.
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13. Zheng X, Gao JX, Zhang H, Yin L, Liu Y, **Zheng P**. Distinct thymic cell types expressing peripheral tumor antigen determine the range and degree of clonal deletion of T cells. 31st Autumn Immunology Conference, November 23 - 25, 2002, Chicago, Illinois.
14. Yang TY, McNally BA, Ferrone S, Liu Y and Zheng P. RNA instability in TAP-1 gene: a potential novel mechanism for tumor evasion of host immunity. 31st Autumn

Immunology Conference, November 23 - 25, 2002, Chicago, Illinois

Funding applied:

PC020104, Rescuing high avidity T cells for prostate cancer immunotherapy.

PI: Pan Zheng, Recommended for funding as "Idea Development Award".

Personnel:

- Pan Zheng, MD, Ph.D., Principal Investigator. Assistant Professor, Division of Cancer Immunology, Department of Pathology, The Ohio State University. 25% effort.
- Xingcheng Zheng, Graduate Research Assistant, Division of Cancer Immunology, Department of Pathology, The Ohio State University. 50% effort (the maximum allowed by graduate student).
- Huiming Zhang, Research Associate, Division of Cancer Immunology, Department of Pathology, The Ohio State University. 50% effort.

(8) Conclusions:

This is the Final report on the grant "Proto-oncogene PML and tumor evasion in prostate cancer". The purpose of the grant proposal is to identify the molecular mechanisms of tumor evasion of host anti-tumor immunity. We have successfully completed the study and a new project has been launched based on the solid preliminary data generated by the support of this grant.

We proposed to identify the antigen presentation defects in human prostate cancer samples and to use mouse prostate cancer model (TRAMP mice) to study the immune regulation and immune tolerance in prostate cancer. We have shown that proto-oncogene PML and the major histocompatibility antigen HLA class I are concordantly down-regulated in high grade prostate cancer. Using mouse prostate cancer model, we have shown that thymic clonal deletion is a major mechanism for immune tolerance to tumor antigens that previously regarded as prostate specific. We provided the direct evidence that the T cell repertoire specific for tumor antigens can be shaped by negative selection in the thymus. During the process of examine antigen presentation defects in different tumor cell lines, we identified a new novel mechanism for antigen presentation gene regulation, i.e. the degradation of mRNA of an antigen presentation gene was involved in tumor evasion of immune recognition. We have also analyzed the transcription regulation of one of the antigen presentation genes and identified two new promoter regions and the essential role of the interferon response factor-binding element (IRFE) in that promoter region. Finally, our preliminary data from chimera mice suggested that distinct thymic cell types expressing peripheral tumor antigen have different roles in determining the range and degree of central and peripheral tolerance. We believe that we have made important contribution to the understanding of immune regulation in prostate cancer and other tumors.

Based on our studies supported by this award, we submitted an "Idea Development Award" proposal entitled **"Rescuing high avidity T cells for prostate cancer immunotherapy"** (PC020104).

Most tumor antigens have the same sequences as the endogenous genes. In addition to their over-expression in the tumors, essentially all of these antigens are expressed in some normal tissues, which is analogous to the so-called peripheral antigen. Most recent studies showed that many of the peripheral antigens are expressed in specialized cells in the thymus, and can induce central tolerance of their specific T cells. However, it is not clear whether mechanisms responsible for tolerizing peripheral antigen in the thymus are responsible for tolerizing potential cancer-specific T cells. We have recently taken a double-transgenic approach to study the mechanism of immune tolerance to a transgenic antigen, SV40-large T antigen (Tag), in the transgenic adenocarcinoma in mouse prostate (TRAMP) mice. Our preliminary data showed that central tolerance plays an important role in immune tolerance to TRAMP mice. In addition, we have published strong evidence that blockage of T cell costimulatory pathway can break the central tolerance for a large array of autoreactive T cells. These two novel observations form the foundation of the current proposal.

Our hypothesis is that blockade of the T cell costimulatory pathway will inhibit the deletion of high avidity tumor antigen specific T cells. The rescued high avidity tumor-antigen specific T cells can respond effectively to prostate cancer cells and delay the development of prostate cancer in the TRAMP model.

We proposed to: (1). Identify the cells in thymus that express peripheral tumor antigen to induce clonal deletion of tumor antigen reactive T cells. (2). Examine whether anti-B7 antibody treatment in TRAMP mice can rescue the tumor-antigen specific T cells that are otherwise

deleted. (3). Determine the thymic function in prostate cancer patients undergoing hormonal therapy.

Various ideas and methods have been developed to augment anti-tumor immunity. Most of the therapies aim at activation of T cells that are already present in peripheral T cell repertoire. However, our preliminary data provide definitive evidence that high avidity tumor antigen specific T cells are deleted through central tolerance. As such, the existing approaches aim at expanding what is likely to be low avidity tumor reactive T cells. The new proposal will explore an innovative idea that high avidity tumor antigen specific T cells can be rescued from clonal deletion by blockade of T cell costimulatory pathway. We anticipate that the rescued T cells will be much more powerful in the combat against tumor. The relevance of our study is further increased by two important factors. First, utilization of spontaneous prostate cancer model will allow us to build a solid experimental foundation for a novel immunotherapy of prostate cancer. Second, the widely used hormonal therapy of prostate-cancer patients may create a new wave of T cell thymopoiesis among them. The random gene re-arrangement

(9) References:

None.

Concordant Down-regulation in Expression of Proto-oncogene PML and the Major Histocompatibility Antigen HLA class I in High-Grade Prostate Cancer

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Abstract

Recognition of tumor cells by cytolytic T lymphocytes depends on expression of cell surface MHC class I. As a mechanism for evasion of T cell recognition, many malignant cancer cells, including those of prostate cancer, have down-regulated MHC class I. For the majority of human cancer, the molecular mechanism of MHC class I down regulation is unclear, although it is well established that MHC class I down-regulation often associates with multiple genes devoted to antigen presentations. Since proto-oncogene PML controls multiple antigen-presentation genes in some murine cancer cells, we analyzed expression of proto-oncogene PML and MHC class I in high-grade prostate cancer. We found that 30 of 37 cases (81%) of Gleasons' grade 7-8 prostate adenocarcinoma have more than 50% down-regulation of HLA class I expression. Among them, 22 cases (73.3%) had undetectable PML protein, while 4 cases (13.3%) showed partial PML down-regulation. In contrast, all 7 cases of prostate cancer with high expression of cell surface HLA class I have high levels of PML expression. Concordant down-regulation of HLA and PML is observed among all different histological patterns of prostate adenocarcinoma. These results suggest that in high-grade prostate cancer, malfunction of proto-oncogene PML is a major factor for down-regulation of cell surface HLA class I, the target molecules essential for direct recognition of cancer cells by cytolytic T lymphocytes.

Introduction

The cytotoxic T lymphocytes (CTL) are the major effector cells against malignant cancer cells (1, 2). The CTL recognize antigenic peptides presented by the human leukocyte antigen (HLA) class I (3) and cause cognate destruction of cancer cells in vivo (4). For this reason, a major focus of cancer immunotherapy is to induce high numbers of CTL specific for cancer cells (5). This effort is greatly facilitated by the identification of a large number of cancer-associated antigens and by the understanding of the molecular basis of T cell activation (6-9). However, it has been established that many human cancers down-regulate cell surface expression of HLA class I (10). In several types of cancers, such as small cell lung carcinoma (11), laryngeal carcinoma and breast carcinoma (12, 13), loss of HLA associates with poor prognosis. Down-regulation of HLA class I expression was observed among 34% of primary prostate adenocarcinoma and 80% in metastatic prostate adenocarcinoma in the lymph nodes (14, 15).

Loss of cell surface HLA class I may allow tumor evasion of immune surveillance (16) and in theory may render them unresponsive to immunotherapy aimed at augmenting cancer-specific CTL. It is, therefore, of great interest to identify molecular basis of HLA down-regulation. Cell surface expression of HLA requires coordinated expression of several antigen-presentation genes (17-20), such as transporter associated with antigen-processing (TAP)-1 and 2, low molecular protein (LMP) 2 and 7, and a TAP-associated protein Tapasin. These

gene products assist in the production, transport and loading of the antigenic peptide with HLA heavy chain and β -2 microglobulin (β 2M). In selected cases, the molecular defects of antigen-presentation, including mutations in β 2M, HLA heavy chain, TAP-1 and TAP-2 have been reported (21-24). However, the mechanisms involved in down-regulation of MHC class I are largely unknown for most cancer cases.

Interestingly, down-regulation of cell surface HLA associates with the malfunction of multiple antigen-processing genes (25-27). This raised an intriguing possibility that molecule(s) that coordinate expression of the antigen-processing genes are defective among the malignant cancer cells. We have previously reported in a murine tumor model that proto-oncogene PML can control expression of multiple antigen-presentation genes and that malfunction of PML is responsible for coordinated down regulation of multiple antigen presentation genes and tumor evasion of T cell immunity (16). The relevance of this observation among human cancer was unclear. An important question is whether HLA loss in human cancer associates with loss of PML expression. To address this question, we analyzed 37 cases of high-grade prostate cancer of different histological patterns. Our data indicate strong association between loss of PML expression and down regulation of cell surface HLA in prostate cancer.

Materials and Methods

1. Antibodies and specimen

Thirty-seven surgically removed prostate adenocarcinoma samples from radical prostatectomy were selected in this study. The specimen were fixed in 10% formalin, embedded in paraffin and processed for routine pathological examination. All cases have been diagnosed and classified according to the Gleasons' Grading. Histological patterns included small acinar, microacinar, cribriform and mixed (cribriform with small acinar and microacinar).

Two antibodies were used for the immunohistochemical analysis. PML-specific monoclonal antibody PG-M3 was purchased from Santa Cruz Biotechnology (Santa Cruz, CA), while HC10 (28), which reacted with HLA-B and C in paraffin section were kindly made available by Dr. Hidde Ploegh (Harvard Medical School, Charles Town, MA) and Dr. Saldano Ferrone (Roswell Park Cancer Institute, Buffalo, NY).

2. Immunohistochemistry

Paraffin sections of 5 μ m each were deparaffinized through graded ethanol solution. After 30 min of antigen retrieval procedure using the target retrieval solution (DAKO, Carpintera, CA), the sections were stained using the avidin-biotin complex system (Vector Laboratory, Burlingame, CA). Step-one reagents are mouse antibodies specific for either HLA or PML. The biotinylated

horse anti-mouse antibodies and horse-radish peroxidase-ABC system (Vector) were used as second and third-step reagents. For single-color staining, 3, 3'-diaminobenzidine (DAB) was used as substrate. We also carried out two-color immunohistochemistry using different substrates. Briefly, the tissue sections were stained with anti-PML mAb, whose binding was detected by the ABC system using the VIP kit (Vector) as the substrate. The cell bound antibodies and enzymes were removed by acid treatment (1N hydrochloride acid for 10 min). After extensive washes, the HC10 antibody was added, and the bound antibodies were detected again using the ABC system using DAB as substrate.

To evaluate the extent of HLA or PML loss, we examined all tumor cells under light microscope and determined the approximate % of tumor cells that are positive for HLA or PML expression. The expression was scored based on the following criteria: >90%, +++; 50-90%, ++; 25-50%, +; 5-25%, \pm ; and <5%, -.

Results and Discussion

1. Down regulation of HLA in prostate cancer tissues

We used monoclonal antibody HC10, which binds the cytoplasmic domain of HLA-A, B and C to evaluate HLA-expression in paraffin sections. Of the 37 cases examined, 14 cases had undetectable amounts of HLA, 16 cases lacked HLA on more than 50% of cancerous cells, while 7 cases had normal HLA on essentially all cancerous tissues (Table 1). Our results are consistent with previous reports on the subject (14, 15).

Four cases of prostate cancer shown in Fig. 1 revealed different patterns of HLA down regulation. Fig. 1a depicted prostate adenocarcinoma cells that expressed normal HLA level. In the case depicted in Fig. 1b, some adenocarcinoma cells showed normal HLA expression, while other tumor glands showed complete loss of HLA expression. The HLA⁺ and HLA⁻ cancerous cells can be found in distinct clusters adjacent to each other. Fig. 1c and 1d are examples of loss of HLA expression in entire cancerous regions. In Fig. 1c, a Gleason grade 3 carcinoma comprising a well-circumscribed cribriform tumor mass was lack of HLA expression while the adjacent benign prostate glands, blood vessel endothelial cells, and scattered fibroblast cells still expressed high level of HLA. Fig. 1d showed an example of individual tumor glands that completely lack of HLA expression. The right upper corner was a benign gland with normal HLA expression.

The existence of cancerous cluster with different degree of HLA loss indicated that lack of HLA on prostate cancer cells is unlikely due to transformation of HLA- precursor cells. Since loss of MHC class I confer an advantage to tumor cells in the presence of T cell response (29), it is likely that HLA loss in cancer cells is due to selection by the immune system.

2. Down-regulation of PML expression on cancerous tissues: association with HLA down-regulation

Since HC10 react with HLA-B and C at both alleles, the HLA loss observed here is likely due either to abnormality of critical antigen presentation

genes such as TAP-1 and TAP-2, or due to malfunction of genes that co-ordinate multiple antigen-presentation genes, such as PML (16). To evaluate this possibility, we use immunohistochemistry to evaluate the PML expression. PML resides primarily in the nucleus and form a characteristic POD domain (30). As shown in Table 1, among the 30 cases with either complete or partial loss of HLA expression, 22 cases lacked detectable PML, while 4 cases had partial loss of PML. PML expression is high on normal prostate tissue and other non-cancerous cells, such as vascular endothelial cells.

A strong correlation between the PML down regulation and HLA loss was observed. As summarized in Table 1, of the 30 cases that showed either complete or partial HLA down-regulation, 26 cases show either complete or partial loss of PM. Conversely, all seven cases with normal HLA expression, PML expression was normal. The correlation between PML expression and expression of HLA on the cell surface became more striking when distribution of HLA⁺ and PML positive cells are considered. As shown in Fig 2a and b, a section of case 31 consisted of HLA⁺ vascular endothelial cells and PIN. The cells that express HLA showed typical nuclear staining of PML, while the HLA-cancer cells are devoid of PML. Prostate epithelial cells in PIN expressed high level of HLA. Correspondingly, strong nuclear staining of PML was found in the cells residing in the PIN. The correlation between PML and HLA also holds in malignant cancer cells that express HLA (Fig. 4). To unequivocally demonstrate the co-expression of PML and HLA, we carried out two-color immunohistochemistry using prostate section with different pattern of HLA

expression and PML expression. As shown in Fig. 5, PML and HLA staining were found in distinct location within the same cells regardless of the stage of cancer. More strikingly, in cases where the HLA⁺ and HLA⁻ cells exist as micro-chimerism, the correlation between HLA and PML also held. Thus, as shown in Fig. 6, a cluster of the HLA⁺ cells surrounded a patch of HLA⁻ cells. All of the HLA⁺ cells with defined nuclei also stained strongly for PML. In contrast, essentially all of the HLA⁻ cells were devoid of PML. These results strongly suggest that PML down-regulation may be involved in HLA down-regulation in the prostate cancer.

Nevertheless, it should be pointed out that we have found 4 cases of prostate cancer that showed partial HLA loss without down regulation of PML. Malfunction of PML therefore cannot be responsible for HLA loss in these 4 cases. It is worth investigating whether other described molecular mechanisms, such as mutation of TAP-1 and TAP-2, β 2M is responsible for HLA loss (21-24).

Although down regulation of PML in neoplastic tissues, especially the malignant ones have been demonstrated (31, 32), our study appears to be the first that show concordant down-regulation of PML and HLA loss. Our results support a critical role of PML defects in HLA loss in prostate cancer. Since HLA loss is a general phenomenon in many cancer types (33), it is worth studying whether these correlation can be extended to other tumor types. Since HLA plays an essential function for presentation of antigens to CTL, down-regulation of HLA may confer tumor resistance to host immunity and adoptive CTL therapy.

Understanding the molecular defects may help us to develop molecular approaches to correct these defects.

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Table 1. Cases number, Gleason Grades, histological pattern and expression of HLA class I and PML of samples used in this study

Surgical Pathological #	age	Gleason Grades	Histological Pattern	HLA class I Expression	PML Expression
978489	68	3+4=7/10	Microacinar	–	–
981293A7	75	3+4=7/10	Small acinar	–	–
9816272L	47	3+4=7/10	Small acinar with cribriform	–	–
982266C13	69	3+4=7/10	Small acinar	–	–
987017C8	64	3+5=9/10	Microacinar	–	–
9816557AA	63	3+4=7/10	Small acinar	–	–
9820348W	51	3+4=7/10	Small acinar with Micro.	–	–
9812396C9	55	3+4=7/10	Small acinar	–	–
98590C7	70	3+4=7/10	Small acinar	–	–
982285C8	55	3+4=7/10	Microacinar with cribriform	–	–
9810389C12	73	3+4=7/10	Small acinar	–	–
984970C014	61	3+4=7/10	Small acinar	–	–
987768	64	3+4=7/10	Small acinar	–	–
965666	50	3+5=8/10	Cribriform	–	–
9816744U	76	3+4=7/10	Small acinar	+	–
9710836	68	3+4=7/10	Cribriform	±	–
985808	50	3+5=8/10	Cbriform	±	–
98498C7	64	3+4=7/10	Small acinar	±	–
9811259C12	64	3+4=7/10	Small acinar	±	–
9822646CS	62	3+4=7/10	Small with cribriform	±	–
984308C8	52	3+4=7/10	Smallµacinar	±	–
9820505	59	3+4=7/10	Small with microacinar	±	–
984647	70	3+4=7/10	Microacinar	±	±
9810081AB	66	3+4=7/10	Small&cribriform	±	±
984715	58	3+4=7/10	Microacinar	±	±
98133C13	69	3+4=7/10	Small acinar		
9817373AC	55	3+4=7/10	Small acinar	+++	+++
978278	74	3+4=7/10	Microacinar	+++	+++
9815476AB	66	3+4=7/10	Small acinar	+++	+++
988569C9	62	3+4=7/10	Small acinar	+++	+++
9820004Q	56	3+4=7/10	Small acinar	+++	+++
981237C8	54	3+4=7/10	Microacinar	+++	+++
986118C18	72	3+4=7/10	Microacinar	+++	+++
985906	65	3+5=8/10	Microacinar	±	+++
9814763C15	74	3+4=7/10	Small acinar	+	+++
9410079E	83	3+4=7/10	Cribriform	±	+++
9818465T	73	3+4=7/10	Small acinar	+	+++

>90% +++ ; >70-90% ++; >50-70% + ; >20-50%±; <20% –.

Table 2. Coordinated expression of PML and HLA in prostate cancer

Concordant down regulation of HLA and PML	81% (26/30)
Concordant expression of PML and HLA	100% (7/7)
Defective HLA expression in the absence of PML Down-regulation	19% (4/30)
Defective PML with normal HLA expression	0% (0/26)

Figure legends

Fig. 1. Different degree of HLA loss among prostate cancer cells. a. Normal level of HLA expression. b. Expression of HLA on vascular endothelial cells and PIN, but not on cancer tissue with single cell infiltration. c and d. Histology (c) and expression of HLA on some but not other clusters in a case of cancer tissues.

Fig. 2. Coordinated expression of HLA and PML in prostate cancer case 31. Upper left, H&E 100x, note the existence of PIN and malignant cancer cells in the same section. Upper right, expression of HLA in PIN and vascular endothelial cells but not malignant cancer cells. Lower right, similar distributions in expression of PML and HLA (100x). Lower left, a 400x micrograph from the same section depicting the nuclear localization of PML expression in vascular endothelial cells.

Fig. 3. Coordinated expression of HLA and PML in PIN. A. H&E section (100x). B. HLA expression (100x). C. HLA expression (400x). D. PML expression (400x).

Fig. 4. Coordinated expression HLA and PML in a case of HLA+ prostate cancer. Note the similar tissue distribution of cells expressing PML and that expressing HLA.

Fig. 5. Downregulation of HLA and PML protein expression in prostate adenocarcinoma. Example of one set of paired serial sections from formalin fixed paraffin embedded prostatectomy sample. Left, 400x, H&E stain; Right, 400x, double stains with HC10 as primary antibody in brown color and with PG-M3 as primary antibody in purple color. Sections A, B show prostate intraepithelial neoplastic (PIN) change, demonstrating expression of both HLA and PML. Sections C, D show intermediate grade prostate adenocarcinoma (Gleason score 3, small gland formation), demonstrating partial simultaneous expression of HLA- and PML on same cells. Section E, F show high grade prostate adenocarcinoma (Gleason score 5, single cell infiltration), demonstrating simultaneous loss of expression of HLA and PML on tumor cells. Arrows mark the positively stained endothelial cells in blood vessel.

Fig. 6. Correlation between HLA and PML expression revealed by two color staining using tissue with mixed HLA expression pattern. Note that a patch of HLA⁻ cells are surrounded by a cluster of HLA⁺ cells. Essentially all of the HLA⁻ cells were negative for PML, while all HLA⁺ cells with definable nuclei showed characteristic PML expression.

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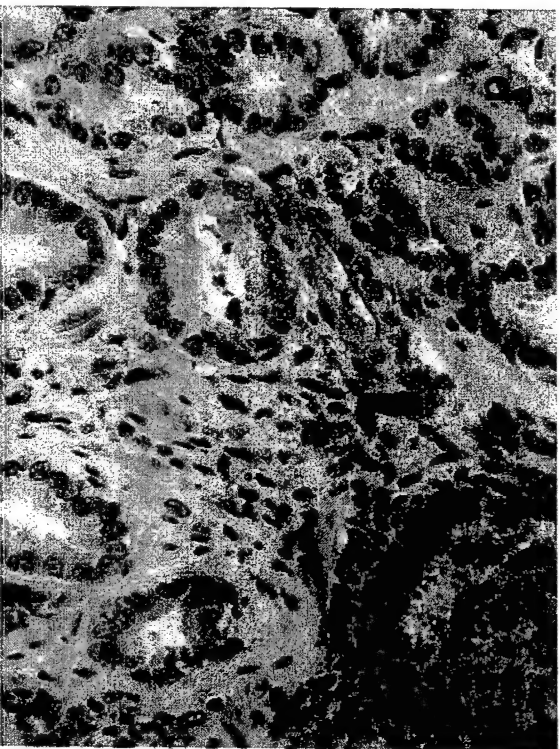
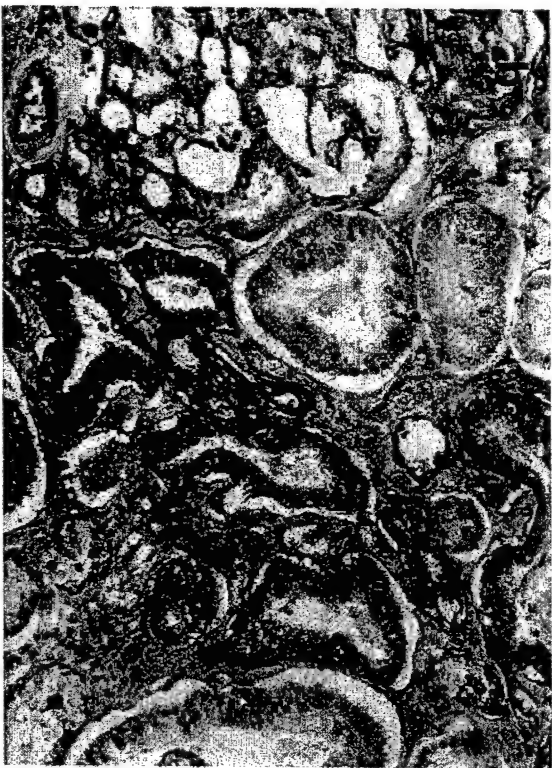
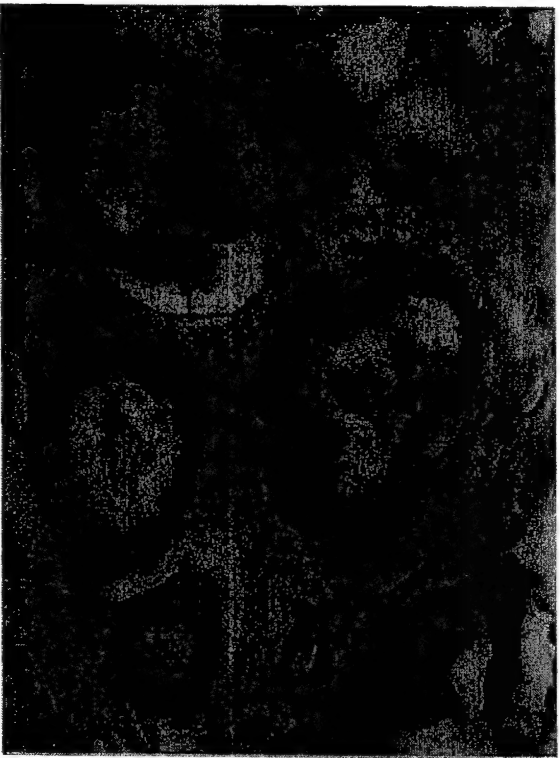


Fig. 1.

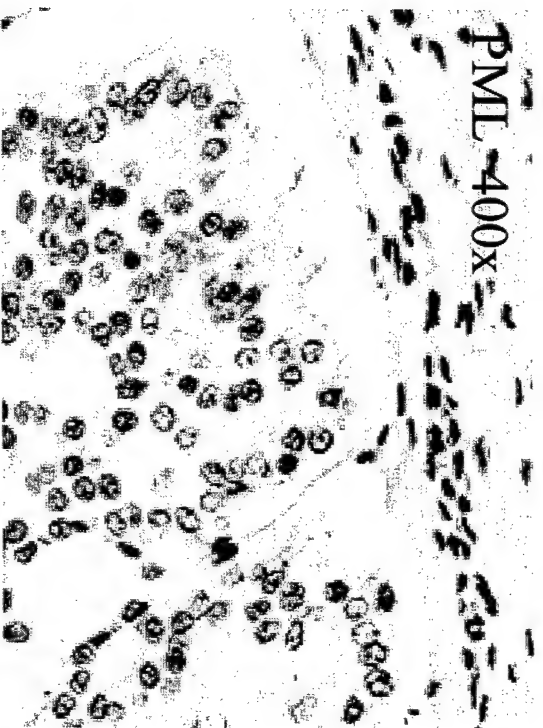
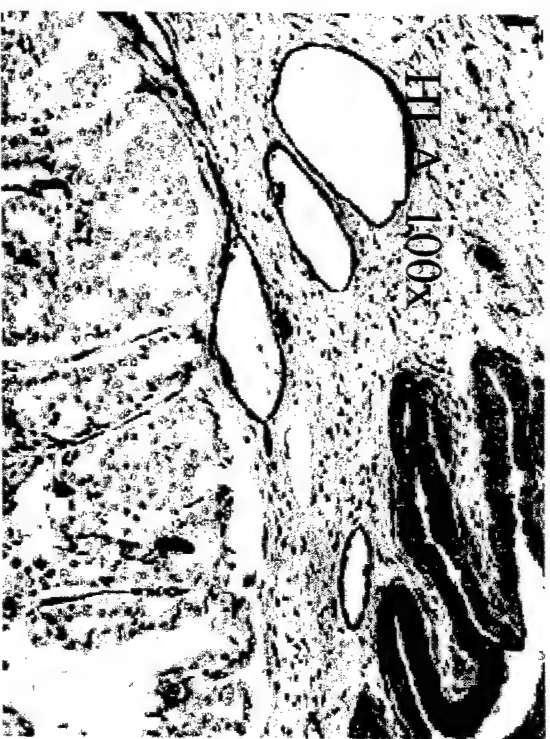


Fig. 2

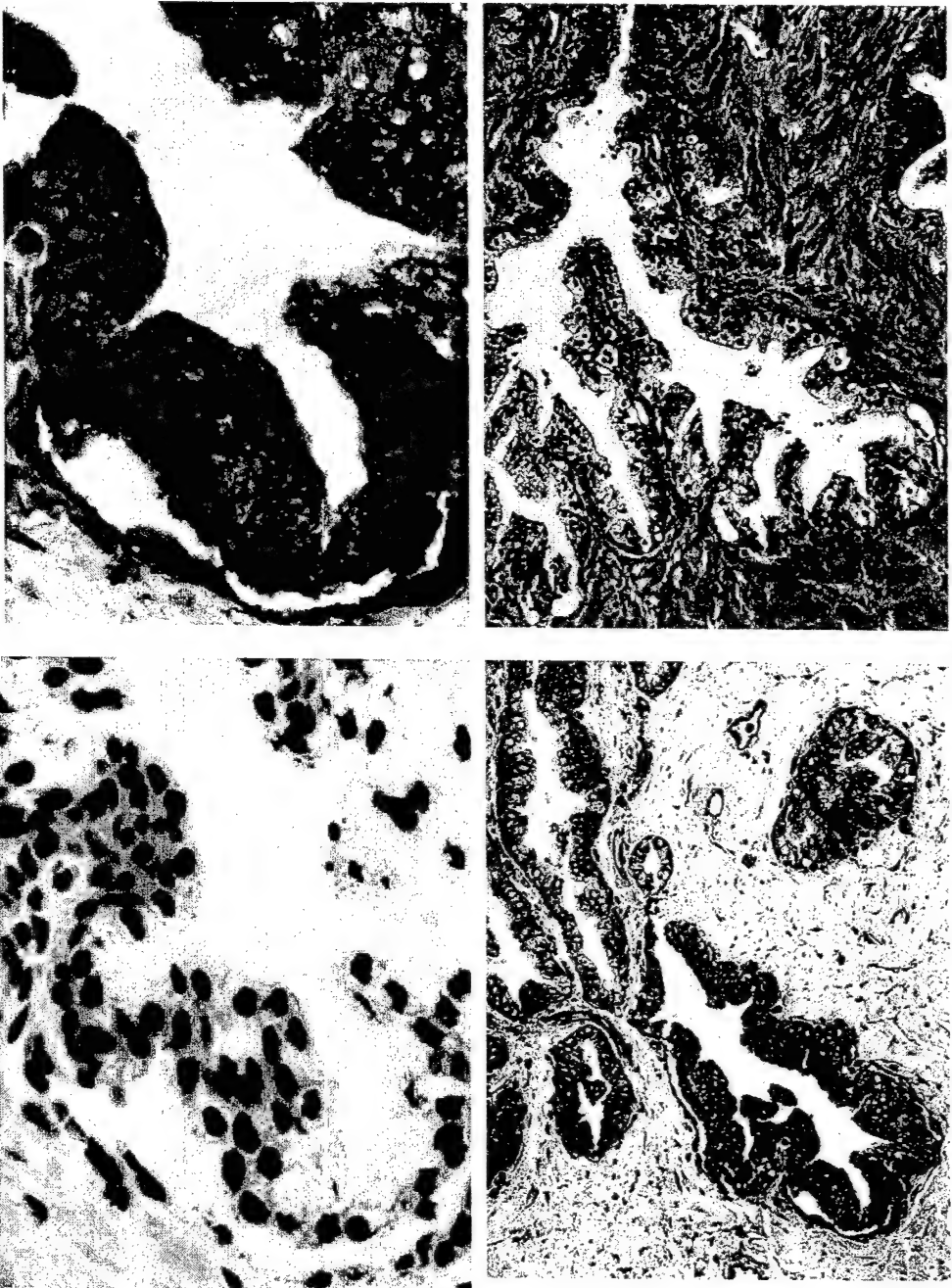
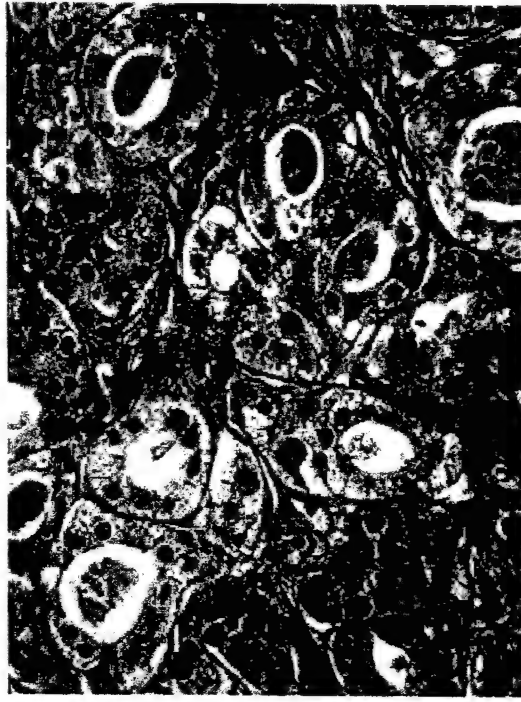
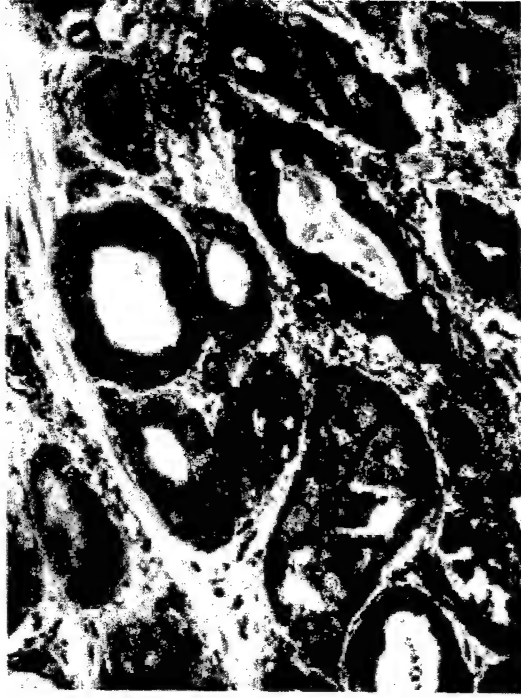


Fig. 3

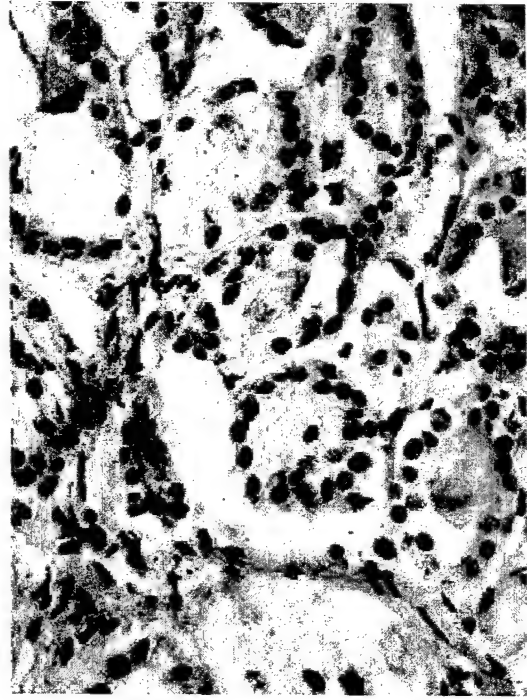
C19, H & E 200x



HLA 200x



PML 200x



PML 400x

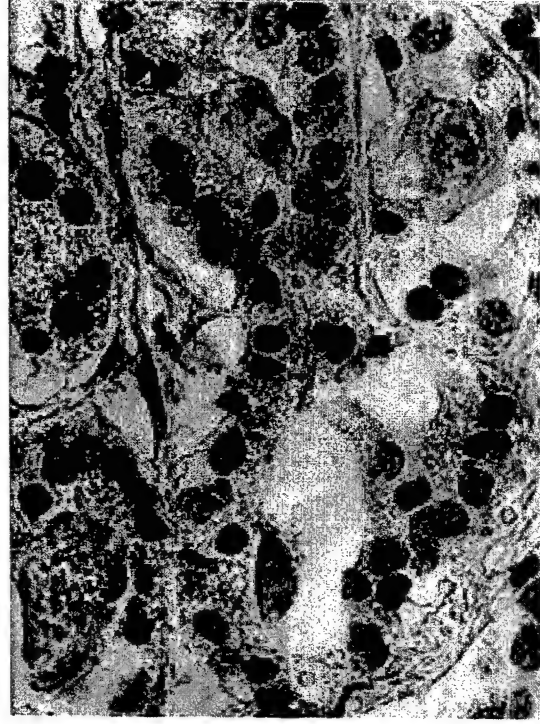


Fig. 4

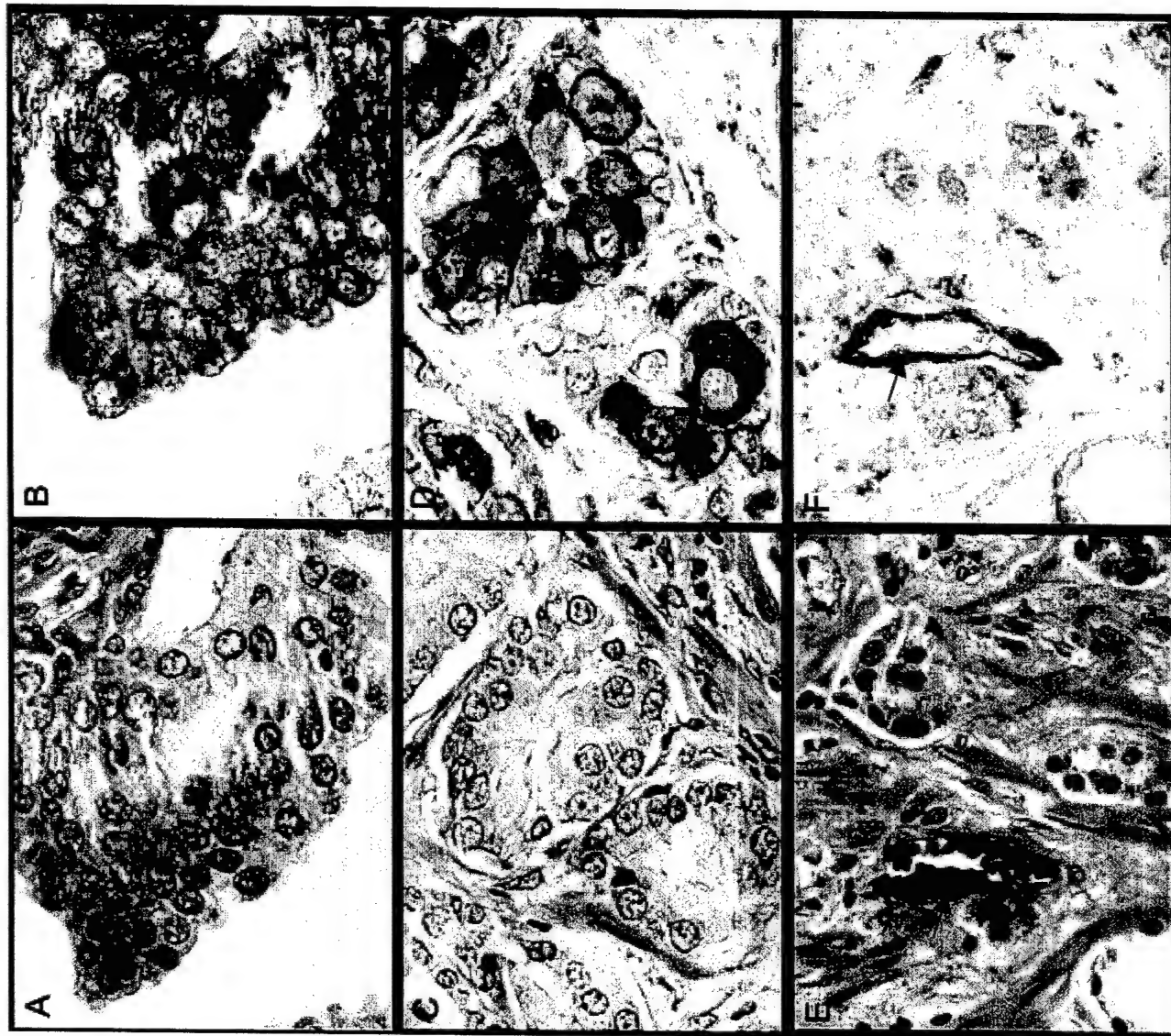


Fig. 5



Fig. 6

**A single nucleotide deletion leads to premature termination codons and degradation of
TAP-1 mRNA: a potential novel mechanism for tumor evasion of host immunity**

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Running Title: mRNA instability and TAP-1 down-regulation in tumor

Summary

Both viruses and tumors evade cytotoxic T lymphocyte (CTL)-mediated host immunity by down-regulation of antigen presentation machineries. The mechanisms underlying these abnormalities include down-regulation of transcription of antigen presentation genes and post-translational inactivation of the proteins involved in antigen presentation. So far no evidence is available to support a post-transcriptional regulation of genes involved in antigen presentation in tumors or viral infected cells. In this study, a major histocompatibility complex (MHC) class I deficient melanoma cell line SK-MEL-19 was found deficient in the expression of the transporter associated with antigen processing (TAP)-1 mRNA even after interferon-gamma (IFN- γ) stimulation, despite its active transcription of the TAP-1 gene. This abnormality is caused by a single nucleotide deletion at position +1489 of the TAP-1 gene, which results in downstream premature termination codons (PTCs) and degradation of the TAP-1 mRNA. To our knowledge, this is the first evidence that the degradation of mRNA of an antigen presentation gene is involved in HLA class I down-regulation in malignant cells, and presumably involved in tumor evasion of recognition and destruction by cytotoxic T lymphocytes.

Introduction

Recent studies demonstrate that patients with malignant melanoma often have high numbers of cytotoxic T lymphocytes (CTL) specific for melanoma-associated antigens (1-3). The co-existence of T cells and tumor cells even in the draining lymph nodes suggests that the tumors were able to evade destruction by host CTL. Accumulating evidence supports the notion that both malfunction of T cells and down-regulation of antigen presentation machinery in tumors can be responsible for tumor evasion of host immunity (4-7). In fact, a high proportion of malignant tumors, including melanoma, have severely depressed cell surface expression of class I HLA antigens, the target molecules that present tumor antigenic peptide to CTL (7). Understanding the mechanisms of the T cell malfunction or antigen presentation defects may thus provide insight for immunotherapy of melanoma and other cancers.

Optimal cell surface expression of HLA molecules requires the coordinated expression of several genes, such as transporters associated with antigen processing (TAP)-1/2, low molecular weight peptide (LMP)-2/7, tapasin, as well as HLA class I heavy chain and β_2 -microglobulin (β_2 M). In cases of both tumorigenesis and viral infection, expression of these genes and the function of the encoded proteins are often down-regulated (7,8). The mechanisms for such down-regulation have been studied extensively. Theoretically, gene expression can be modulated by transcriptional, post-transcriptional, translational and post-translational mechanisms. The mechanisms that have been shown to underlie the antigen presentation abnormalities are either transcriptional suppression of antigen presentation genes, and/or functional inactivation of their gene products, either by missense mutation or by protein-protein interactions (9-12). Here we show that actively transcribed TAP-1 mRNA in the melanoma cell line SK-MEL-19 is rapidly degraded even after stimulation with IFN- γ . Cloning and sequencing

analysis have revealed that this rapid mRNA degradation is caused by a single nucleotide deletion resulting in a pre-mature termination codon (PTC). These results reveal a new potential mechanism for tumor evasion of host T cell recognition.

Experimental procedures:

Cell lines and antibodies: Human melanoma cell lines 1195, 1102 and SK-MEL-19 have been cultured as described (13). The breast cancer cell line SK-BR-3 was obtained from ATCC (HTB-30, ATCC, Manassas, VA.). All the cells were grown in DMEM supplemented with 10% heat-inactivated fetal calf serum (FCS), 2mM L-glutamine, 100 IU/ml penicillin and 100 µg/ml streptomycin (Life Technologies, Inc., Grand Island, NY). For induction of HLA class I expression, cells were cultured in medium supplemented with recombinant human IFN-γ (1000 U/ml) (R&D Systems, Inc., Minneapolis, MN). PE-conjugated anti-HLA-A, B, C antibody (clone G46-2.6) and isotype control PE-conjugated mouse IgG1 were purchased from BD PharMingen (San Diego, CA).

Flow cytometry: HLA class I expression on the cell surface of all cell lines was examined by flow cytometry as described (5). Briefly, viable cells were incubated with PE-conjugated mouse IgG1 and PE-conjugated anti-HLA-A, B, C antibody at 4°C for 2 h. After three washes with PBS containing 1% FCS, cells were fixed with 1% paraformaldehyde and examined by flow cytometry.

Northern blot: The cells were treated with IFN-γ (R&D Systems, Inc.) at 1000 U/ml for 48 hrs or untreated. For cycloheximide (CHX) (Sigma Chemical Corp., St. Louis, MO) treatment, SK-MEL-19 cells were cultured with IFN-γ at 1000 U/ml for 48 hours and then CHX were added to the cells for final concentrations of 5 µg/ml or 10 µg/ml, respectively, for up to 16 h. Total RNA was isolated using TRIzol reagent (Life Technologies, Inc.). Hybridization conditions followed the instructions of the Northern hybridization kit (Eppendorf Scientific, Inc., Westbury, NY). The cDNA probes for TAP-1, TAP-2, LMP-2, LMP-7, HLA class I heavy chain, and β2M were made from PCR products using primers listed previously (14). Human

splenocyte cDNA library from Invitrogen (Invitrogen Corp., Carlsbad, CA) was used as template for PCR reactions. All PCR products had been subcloned into pBluescript vector (Stratagene Corp., La Jolla, CA), sequenced and confirmed to be identical to published sequences. The probes were labeled with α -[32 P]-dCTP (NEN Life Science Inc., Boston, MA) using the DECAprimeTM II kit (Ambion Inc., Austin, TX).

Generation of TAP-1 cDNA constructs and stable transfection: Human small cell carcinoma H146 cell line (provided by Dr. N.P. Restifo, National Cancer Institute, Bethesda, MD) was incubated with IFN- γ at 1000 U/ml for 48 hours. Total RNA was isolated as above. Reverse transcription was done using the SUPERScript First-Strand cDNA Synthesis System (Life Technologies, Inc.). TAP-1 cDNA was amplified by PCR in three fragments. Primers are: hTAP.f1 5'-GCGGCCGCTTTCGATTTC GCTTTC-3', hTAP.r1 5'-TGCAGTAG CCTGGTGCTATCCG-3', hTAP.f2 5'-CTTGCCTT GTTCCGAGAGCTGA-3', hTAP.r2 5'-CTCGTTGGCAAAGCTTCGAAC-3', hTAP.f3 5'-CGGCCATGCCTACAGTTCGAAG-3', hTAP. r3: 5'-ATAAATATCAAGAACCTACAG GG-3'. The three fragments were cloned into pBS-KS vector (STRATAGENE, La Jolla, CA) at *NotI*/*SmaI*, *SmaI*/*HindIII* and *HindIII*/*XhoI* sites, respectively, and sequenced to confirm that the cDNA has a wild-type sequence. When the SK-MEL-19 cells grew to 70% confluence in the 24-well plate, 0.2 μ g pcDNA3.1/Hyg(+) vector (Invitrogen Inc.) and pcDNA3.1/Hyg(+) vector with wild type TAP-1 cDNA insert were respectively transfected into each well, using 6 μ l Fugene 6 transfection reagent (Roche Diagnostics Corporation, Indianapolis, IN) according to the manual. 48 hours later, the transfected cells were re-plated onto 96-well plates and cultured in the DMEM medium and in the presence of 0.5 mg/ml hygromycin. Single cell clones were selected for further culture and analyzed for HLA class I antigen expression.

Southern blot: Genomic DNA was isolated from SK-MEL-19 cells, SK-BR-3 cells and HeLa cells. Genomic DNA (20 µg) was digested with *Afl*III (Life Technologies, Inc.) and separated in 0.8% agarose gel. The TAP-1 promoter probe was made by PCR from normal human lymphocyte genomic DNA with sense primer: 5'-TCCCGCCTCGAGCATCCCTGCAAGGCA-3' and anti-sense primer: 5'-TGCAGTAGCCTGGTGCTATCCG-3'. Probes were labeled as described above.

Generation of Luciferase-reporter constructs and assay for promoter activity: TAP-1 promoter was amplified from SK-MEL-19 cell genomic DNA by PCR using the primers: hTAP1.Pr1 5'- GCTCTAGATGGCACTCGGACGCCGTC-3' and hLMP2.Pf1 5'- GCTCTAGACCCTGCAAGGCACCGCTC-3'. The PCR products were sub-cloned using the Zero Blunt TOPO PCR cloning kit (Invitrogen Corp.) and then cloned into pGL2-basic vector (Promega Corp., Madison WI) at *Xho*I and *Hind*III sites. All constructs were confirmed by DNA sequencing. Expression level of the firefly luciferase from the pGL2 constructs (basic, SV40, pTAP1/T and pTAP1/G) was normalized to the internal control pRL-SV40 *Renilla* luciferase level. Results were shown as the folds of increase compared with the pGL2-basic (basic). The dual luciferase assay was carried out according to the manufacturer's instructions (Promega Corp.).

Nuclear run-on assay: The assay was performed as described elsewhere (15). Briefly, nuclei were extracted from 10^7 - 10^8 SK-MEL-19 cells treated with 1000 U/ml of IFN-γ or without any treatment. The transcripts were labeled *in vitro* with 40 nM biotin-16-UTP (Roche Diagnostics Corp.) in the presence of 3.75 mM ATP, GTP and CTP, 25 mM Tris-HCl, 12.5 mM MgCl₂ and 750 mM KCl. cDNA fragments of LMP-2 and GAPDH were amplified by PCR from cloned cDNA constructs (14). TAP-1 cDNA fragment was amplified by PCR from cloned

cDNA constructs using primers hTAP1.f1 and hTAP1.r1 described above. The pcDNA3.1/Hyg(+) vector was linearized with *Hind*III. All the DNA was immobilized on nitrocellulose membrane using S&S Minifold II slot blot apparatus according to the manual (Schleicher & Schuell Inc., Keene, NH). Hybridization condition was as described before (15), and the biotin-labeled transcripts were detected using streptavidin-alkaline phosphatase conjugate (Roche Diagnostics Corporation, Indianapolis, IN) and CDP-star Ready-To-Use with Nitro-Block-II reagent (Tropix Inc., Bedford, MA).

Restriction fragment length polymorphism (RFLP): Primers hTAP1CE7.f 5'-GCACCCCTCGCTGCCTACCCAGTGGTCT-3' and hTAP1E7.r 5'-TACAGGGAGTGGTAGGTTGTACCTG-3' were used to amplify the region in the TAP-1 exon 7 where the single nucleotide deletion resides from genomic DNA. The region was also amplified from cDNA using primers hTAP1E7.f and hTAP1cE7.r. PCR products were separated by gel electrophoresis and purified using QIAGEN gel extraction kit (QIAGEN Inc., Valencia, CA). The purified PCR products were incubated with *Bs*II (New England Biolabs, Inc., Beverly, MA) at 55°C overnight and then separated in 5% agarose gel.

Results and Discussion

Down-regulation of TAP-1 mRNA by a post-transcriptional mechanism in melanoma cell line SK-MEL-19.

Three human melanoma cell lines, 1102, 1195 and SK-MEL-19, were examined by flow cytometry for their cell surface HLA class I expression with or without IFN- γ stimulation. A PE-conjugated anti-human HLA-A, B, C antibody was used to detect all HLA class I alleles, and a PE-conjugated mouse IgG1 was used as isotype control. As shown in Figure 1a, 1102 and 1195 cells had significant HLA class I that was further up-regulated by incubation with 1000 U/ml IFN- γ for three days. Confirming previous studies (13), we found that SK-MEL-19 cells had no cell surface HLA. Surprisingly, while other melanoma cell lines up-regulated their cell surface HLA in response to IFN- γ , very little HLA class I antigen could be found on the SK-MEL-19 even after IFN- γ -treatment.

Since optimal cell surface HLA class I expression requires the coordinated expression of multiple genes, including TAP-1/2, LMP-2/7, β_2M as well as HLA class I heavy chain, a Northern blot analysis was performed to detect the expression of these genes (Fig. 1b). In 1102 and 1195 cells, all six genes were expressed at low but detectable levels. IFN- γ -treatment drastically induced expression of all six genes. Interestingly, in the SK-MEL-19 cells, while β_2M , HLA heavy chain, LMP-2, LMP-7 and TAP-2 were present at low levels without induction, no TAP-1 mRNA was detected. After IFN- γ treatment, β_2M , HLA heavy chain, TAP-2, LMP-2 and LMP-7 were expressed at high levels, yet TAP-1 was still expressed at low levels.

It had been known that TAP deficient cells can express HLA class I after transfection with TAP-1 or TAP-2 gene (16-18). To test whether the lack of TAP-1 expression was responsible for the barely detectable expression of HLA class I antigen on the surface of SK-

MEL-19 cells, we transfected the cells with TAP-1 cDNA. As shown in Fig. 1c, the TAP-1 cDNA transfected SK-MEL-19 cells expressed significant levels of HLA class I antigen even prior to IFN- γ -treatment. Moreover, the TAP-1-transfectants were as responsive to IFN- γ as the other melanoma cell lines. Based on these results, it is likely that the primary defect of antigen presentation in SK-MEL-19 cells is attributable to defects in TAP-1 expression.

Since the TAP-1 expression was low at the mRNA level, the TAP-1 down-regulation may be caused by defective transcription or malfunction in the RNA metabolism. The TAP-1 expression was under the control of a bi-directional promoter, as characterized by Ting and colleagues (19). We cloned and sequenced the 593 base pair TAP-1 promoter from the SK-MEL-19 cells. In comparison to the published sequence (19), a single nucleotide G>T replacement was identified at position -446 (the first ATG of TAP-1 gene is designated as +1), which was close to the first transcription start site at -427 (19) (Fig. 2a). As the T allele resulted in a loss of restriction site *Afl*III, we did a Southern blot hybridization using *Afl*III to confirm the mismatch. As shown in Fig. 2a, while the HeLa cell line contained homozygous G alleles as described (19), both SK-MEL-19 and breast cancer cell line SK-BR-3 were homozygous for T alleles which loss the a restriction site for *Afl*III. To test if this single nucleotide replacement results in decreased promoter activity, both alleles of the TAP-1 promoter were cloned into the pGL2-basic vector that had the luciferase gene as reporter. As shown in Figure 2b, the T allele TAP-1 promoter retained 50% promoter activity of the G allele. However, given the significant variation in transient transfection and luciferase assay, it is unclear that the G>T change has significant effect on TAP-1 transcription. Importantly, both reporters were equally efficiently induced by IFN- γ treatment. Moreover, our analysis of normal human peripheral blood lymphocyte samples revealed that both alleles were present at a high frequency, and individuals

that carry either G or T alleles have equivalent cell surface HLA class I antigen expression (data not shown).

We therefore performed a nuclear run-on assay to directly evaluate the transcription of the TAP-1 gene. LMP-2 transcription, which was under the control of the same bi-directional promoter, was also evaluated. As shown in Fig. 2c, TAP-1 was transcribed at high levels in SK-MEL-19 cells under basal condition, although IFN- γ appears to up-regulate TAP-1 transcription somewhat. In contrast, LMP-2 was transcribed at an undetectable level, but was induced to high levels by IFN- γ (Fig. 2c). The lack of LMP-2 transcription at basal condition may reflect the IFN- γ inducible expression pattern of this gene. These results demonstrated that lack of TAP-1 mRNA in SK-MEL-19 cells was not due to defective transcription. Taken together, the results demonstrate that a post-transcriptional defect is responsible for poor TAP-1 expression in SK-MEL-19 cells even after IFN- γ stimulation. Numerous studies have revealed defective TAP-1 expression among tumor cells (reviewed by reference 4). To our knowledge, however, this is the first example of a post-transcriptional defect of TAP-1 expression.

A single-nucleotide deletion leads to premature termination codons (PTC) and degradation of TAP-1 mRNA

A major mechanism responsible for post-transcriptional regulation of mRNA is RNA degradation, which can be prevented by cycloheximide (CHX), a protein synthesis inhibitor of mammalian cells. It is well established that the turnover of mRNA is closely linked to the translation process and blocking of translation can stabilize mRNA, especially those with short half-lives (20-22). To test if the accelerated RNA degradation is responsible for the lack of TAP-1 in SK-MEL-19 cells, we treated the SK-MEL-19 and control HeLa cells with CHX after

incubation with or without IFN- γ (1000 U/ml) for 48 hours at 37°C. At different time points after the CHX was added to the cell culture, cells were harvested and the total cellular RNA was analyzed for TAP-1 mRNA. The intensity of each band was quantified using the ImageQuant 5.0 software (Molecular Dynamics, Sunnyvale, CA) after exposure to a phospho-image screen. For a better comparison, TAP-1 mRNA levels were normalized to the endogenous house-keeping gene GAPDH level and the folds of increase compared with the non-CHX treated cells were then calculated. Under basal condition, the TAP-1 mRNA was up-regulated by maximal 4.8 folds in SK-MEL-19 cells. After IFN- γ induction, the TAP-1 mRNA was up-regulated by 25.7 folds. In comparison, CHX caused less significant increase of TAP-1 mRNA in both IFN- γ -treated and untreated HeLa cells. Taken together, the lack of TAP-1 mRNA, the normal transcription of TAP-1, and rescue of TAP-1 mRNA by CHX treatment indicate that the TAP-1 mRNA was rapidly degraded in the SK-MEL-19 cells. It is noteworthy that in SK-MEL-19 cells, the effect of CHX was significantly stronger when used in combination with IFN- γ . This finding cannot be fully explained by the fact the IFN- γ is a transcriptional activator for antigen presentation genes, as its effect on TAP-1 transcription is not so obvious in SK-MEL-19 cells as shown in Fig. 2c. It is likely that IFN- γ stabilized mRNA in SK-MEL-19 cells, although this possibility remains to be tested formally.

The rapid degradation of TAP-1 mRNA can be due to a genetic lesion in the TAP-1 gene. Alternatively, it is possible that the tumor cell line expressed factors that can cause TAP-1 mRNA degradation. The successful rescue of cell surface HLA class I antigen expression by wild-type TAP-1 in SK-MEL-19 cells favors the first hypothesis, since a wild type cDNA can be expressed in the tumor cell line. As the first-step to test this hypothesis, we cloned the TAP-1 cDNA from SK-MEL-19 cells that were treated with both IFN- γ and CHX. All the three clones

sequenced showed a single nucleotide deletion at position +1489 (Fig. 4a), which resides in exon 7 in TAP-1 gene. Further analysis showed that multiple downstream PTCs (the closest one is at position +1555) were present due to this nucleotide deletion (two of them were shown in Fig. 4c). To confirm that the mutation is in the TAP-1 gene, we amplified exon 7 of the TAP-1 gene from the SK-MEL-19 cells by PCR. The PCR products were digested with *Bst*I, since this restriction enzyme recognized the deletion mutant but did not recognize the wild-type exon 7. Since complete digestion was obtained, the SK-MEL-19 cells are homozygous for this frame-shift mutation (Fig. 4b), even though the cytogenetic analysis revealed that there are 4 copies of chromosome 6 present in the SK-MEL-19 cells (data not shown). We subsequently amplified exon 7 of the TAP-1 gene from 50 normal human peripheral lymphocyte genomic DNA samples by PCR and subjected the PCR products to *Bst*I digestion. Since none of the PCR products from the 50 samples was digested by *Bst*I, it is most likely that the PTCs in SK-MEL-19 cells were resulted from a somatic mutation (data not shown).

PTCs have been shown to interfere with the metabolisms of many different mRNA in mammalian cells, leading to nonsense mediated altered RNA splicing, such as exon skipping and intron retention, or nonsense-mediated mRNA decay (20-22). Since wild type mRNA was functional in the tumor cell line, the frame-shift mutation we identified is most likely responsible for the low TAP-1 mRNA level in the SK-MEL-19 cells. Moreover, the lack of TAP-1 mRNA is probably due to nonsense-mediated mRNA decay, as CHX increased the TAP-1 mRNA level dramatically (26 folds) and no alternatively spliced TAP-1 mRNA was detected from the SK-MEL-19 cells by either RT-PCR or Northern blot. To our knowledge, this is the first report showing PTC-mediated mRNA degradation of any genes involved in antigen presentation. However, the increased turnover of HLA-C heavy chain mRNA has been suggested to contribute

to the low level of HLA-C surface expression (23). Moreover, nonsense mutation and frame-shift mutation were identified in β_2M and TAP-2 genes in tumor cells and immune deficient patients, respectively (24,25). It is of interest to determine whether these nonsense mutations and frame-shift mutations affect mRNA level in the tumor cells by either nonsense mediated altered RNA splicing or nonsense-mediated mRNA decay.

TAP-1 protein has three major domains: transmembrane core, peptide-binding domain and nucleotide-binding domain (26). Theoretically, the frameshift mutation identified here results in a truncated protein product that lacks the nucleotide-binding domain. However, since the mRNA was degraded, it is unlikely that this protein will be produced in significant quantity. Given the importance of ATP hydrolysis in TAP-1 function, it is of interest to consider whether degradation of the mutant TAP-1 mRNA serves any useful purpose for tumor evasion of CTL recognition. A recent study from Cresswell's laboratory (27) suggested that the nucleotide binding activity of TAP-1 is less important than that of TAP-2. The truncated protein in this case may still be functional. As such, removing the TAP-1 mRNA may be essential for tumor evasion of CTL recognition.

HLA class I down-regulation has been reported in different tumor cells and may be one of the mechanisms for the tumor escape from pre-existing anti-tumor CTLs. TAP-1, as a necessary component in the HLA class I expression pathway, has also been reported to be deficient in different tumor cells (7). In primary melanoma TAP-1 down-regulation has been shown as an independent marker for poor prognosis (28). In metastatic melanoma, the frequency of TAP-1 down-regulation is as high as 83% in one report (29). It is of interest to note that our results may suggest new methods to restore antigen presentation in tumor cells. For example, in cystic fibrosis patients, one common nonsense allele, W1282X, results in unstable

mRNA. Treatment of cells harboring this allele with aminoglycosides, which promotes nonsense suppression, results in the re-expression of cystic fibrosis transmembrane conductance regulator (CFTR) on the cell surface and restoration of a cAMP-activated chloride current (30). Similar strategy might be employed in cancer immunotherapy if there is a nonsense mutation or frame shift mutation in the antigen processing genes in tumor cells that will not completely inactivate the protein.

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Figure legends.

Fig. 1. Deficiency of surface HLA class I expression in melanoma cell line SK-MEL-19 was due to the TAP-1 down-regulation. (a). HLA class I expression in three melanoma cell lines, SK-MEL-19, 1102 and 1195. Bold black lines depict the staining by PE-conjugated anti-human HLA-A,B,C antibody in untreated cells, dotted lines represent the staining by PE-conjugated mouse IgG1 as isotype control, and red lines represent anti-HLA-A,B,C antibody staining after stimulation with 1000 U/ml IFN- γ for 72 hours. (b). Expression of HLA class I heavy chain (MHC I), β_2 M, TAP-1, TAP-2, LMP-2 and LMP-7 in each cell line with or without IFN- γ induction (1000 U/ml for 72 hours). Total RNA loading to each well was shown as 28s rRNA and 18s rRNA. (c). Transfection with wild type TAP-1, but not vector alone, restored the HLA class I expression in the SK-MEL-19 cells. SK-MEL-19 cells were transfected with either vector alone (top panel) or vector with TAP-1 cDNA insert (lower panel). These stable clones from each group were stimulated with or without IFN- γ and analyzed for cell surface HLA-A, B, C, as detailed in (a).

Fig. 2. Post-transcriptional mechanisms are responsible for poor accumulation of TAP-1 mRNA. (a). A single nucleotide polymorphism, adjacent to the first transcription start site (-427), was identified at -446 in the bi-directional promoter shared by TAP-1 and LMP-2 genes. The G>T change results in the loss of *Afl*III restriction site. Southern blot hybridization was performed using *Afl*III and detected by a DNA probe that encompasses the downstream region of the polymorphism site. SK-MEL-19 cells showed one 5.6 kb band that represents homozygous T allele, as did the breast cancer cell line SK-BR-3, which has significant cell surface HLA class I surface expression (data not shown). HeLa cells, in contrast, are homozygous for the G allele.

(b). Activities of T and G alleles of TAP-1 promoter (pTAP1/T and pTAP1/G, respectively) in SK-MEL-19 cells. The two allelic forms of TAP-1 promoter were cloned into the pGL2-basic vector (basic) that does not contain any promoter or enhancer but encodes the firefly luciferase. The pGL2-SV40 construct (SV40) that has both SV40 promoter and SV40 enhancer as well as the firefly luciferase reporter gene was used as the positive control. After transfection, IFN- γ was added to the cell culture at 1000 U/ml. Cells were lysed 48 hours after transfection and luciferase expression was tested using a luminometer. Data shown are representative of at least five independent experiments. (c). The TAP-1 gene was actively transcribed in SK-MEL-19 cells in the presence and absence of IFN- γ : nuclear run-on assay. Endogenous GAPDH expression was used as a positive control and the pcDNA3.1/Hyg(+) vector as a negative control. The run-on experiments have been repeated 3 times with similar results.

Fig. 3. TAP-1 mRNA level in SK-MEL-19 cells was increased by CHX. Protein synthesis inhibitor CHX was added to the SK-MEL-19 cells and HeLa cells that have normal TAP-1 and HLA class I expression. Total RNA was isolated from both cells at different time points and subjected to Northern blot hybridization to detect TAP-1 expression. The blot was exposed to a phosphor-imager and the signal intensity was quantified using ImageQuant 5.0 software (Molecular Dynamics, Sunnyvale, CA). After normalization of TAP-1 signal to endogenous GAPDH signal in each sample, the signals in CHX treated group were compared to those that received no CHX treatment. These signals were quantitated as folds of those in untreated cells.

Fig. 4. A homozygous single nucleotide deletion was identified in the TAP-1 gene at position +1489 that resulted in premature termination codons (PTCs). (a). Sequencing chromatogram.

Arrow points to the deletion site. (b). Primers hTAP1E7.f and hTAP1E7.r (arrows) were used to amplify the deletion region in TAP-1 exon 7 (E7). Arrowhead points to the position of the deletion resulting in a new *Bs*II site. The PCR products of genomic DNA were purified and digested with *Bs*II. Gel electrophoresis data showed that SK-MEL-19 cells were homozygous for the +1489 deletion. U, uncut; B, *Bs*II digested; M, molecular weight. (c). The sequence of the deletion region was shown and the downstream PTCs (X) were underlined.

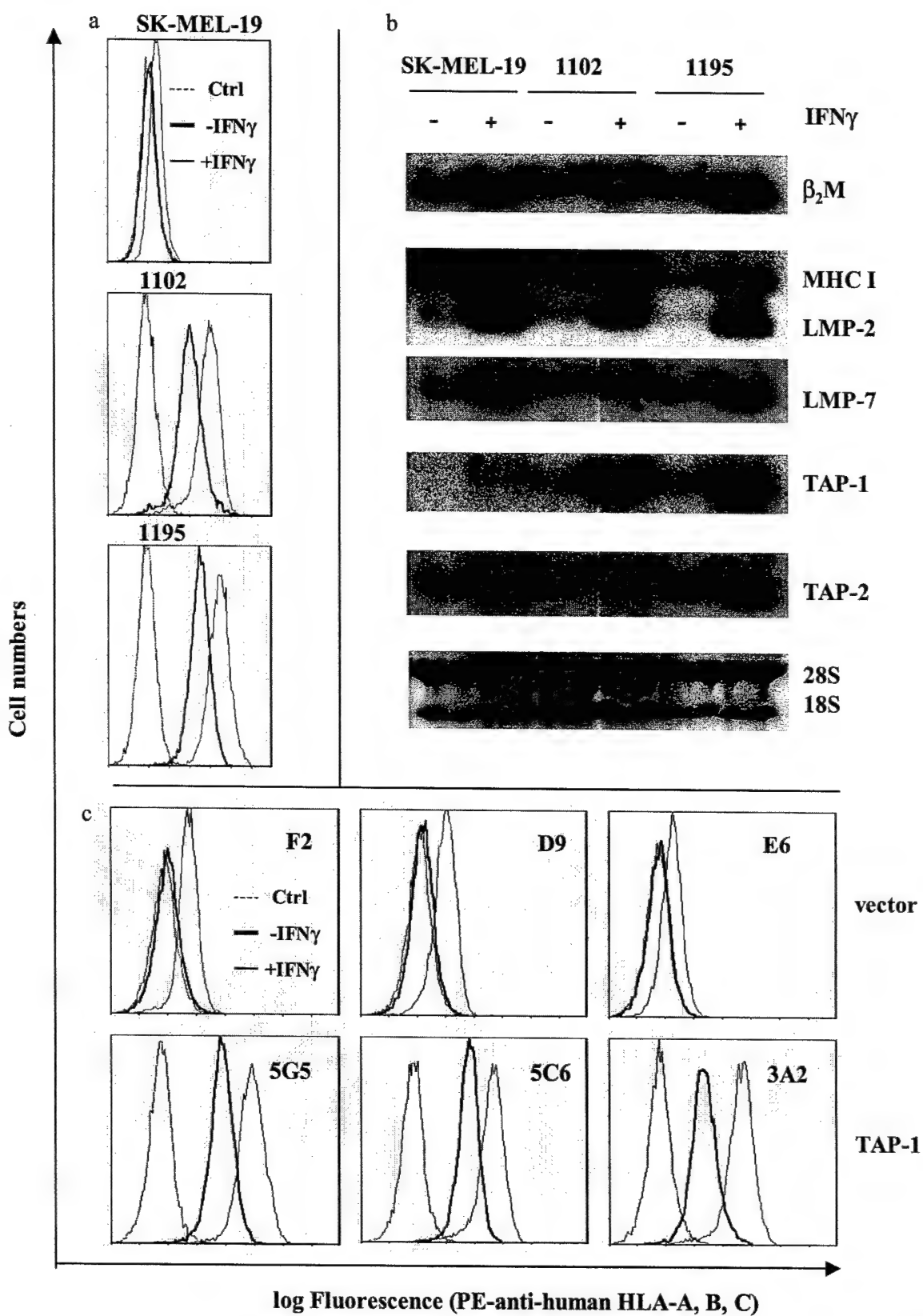


Figure 1

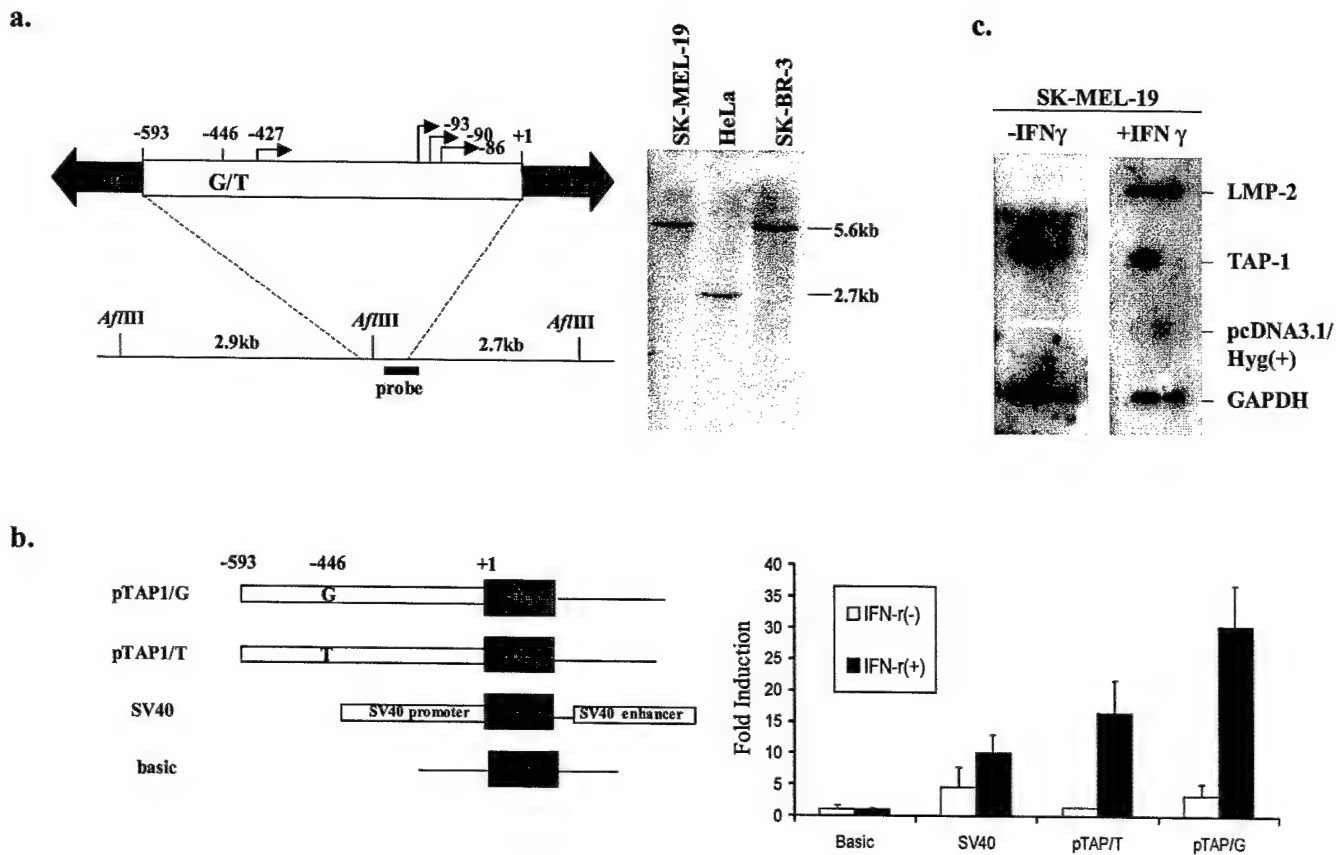


Figure 2

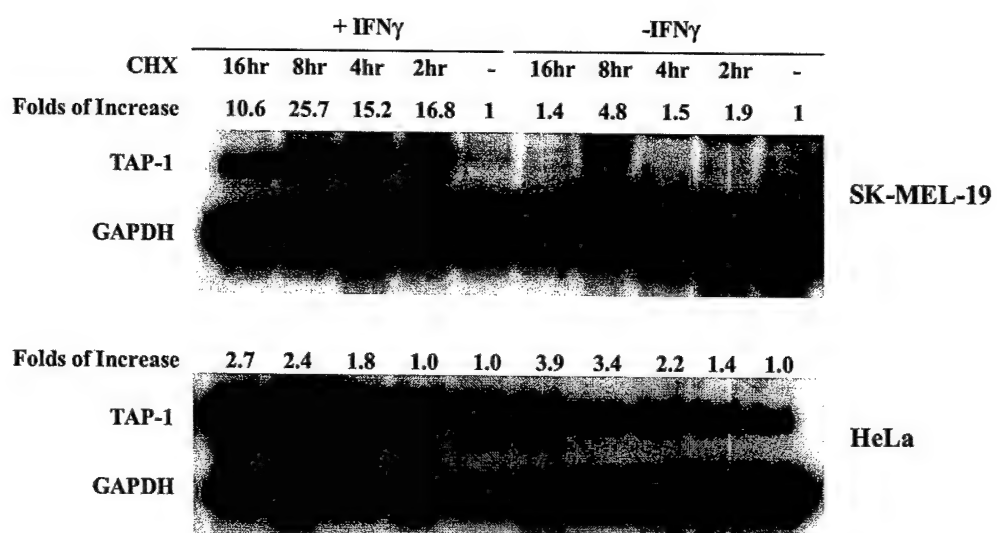
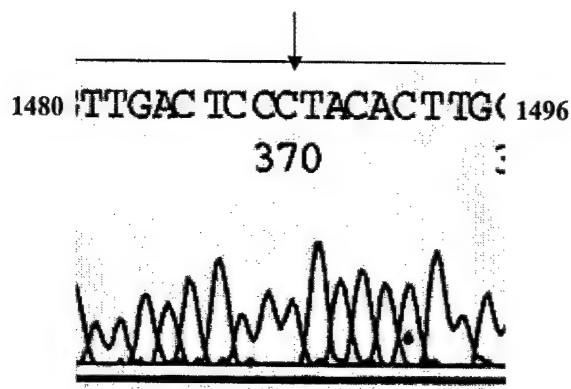
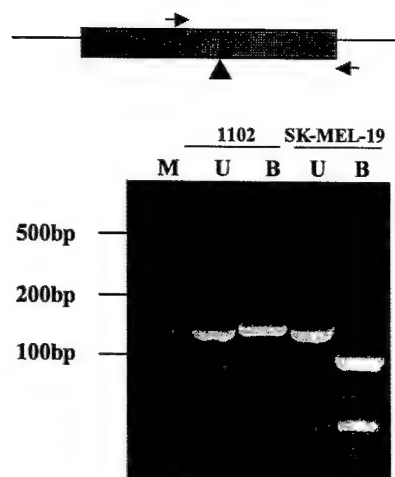


Figure 3

a.



b.



c.

	L T P Y T W R A L S S S K M S P L P T Q T A Q M S X C Y R G X	
Mu: 1480	TTGACTCCC-TACACTTGGAGGGCCTTGTCCAGTTCCAAGATGTCTCCTTTGCCTACCCAAACCGCCAGATGTCTTAGTGCTACAGGGGCTGA	1572
WT: 1480	TTGACTCCCTTACACTTGGAGGGCCTTGTCCAGTTCCAAGATGTCTCCTTTGCCTACCCAAACCGCCAGATGTCTTAGTGCTACAGGGGCTGA	1573
	L T P L H L E G L V Q F Q D V S F A Y P N R P D V L V L Q G L	

Figure 4

Cis elements for transporter associated with antigen-processing-2 transcription: two new promoters and an essential role of the IFN response factor binding element in IFN- γ -mediated activation of the transcription initiator

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Keywords: antigen presentation genes, IFN- γ activation, transporters associated with antigen-processing-2, transcriptional regulation

Abstract

Expression of cell surface MHC class I:peptide complex requires coordinated expression of multiple genes such as MHC class I heavy chain, β_2 -microglobulin (β_2m), transporters associated with antigen-processing (TAP)-1 and TAP-2, and proteosomal components low-molecular weight polypeptide (LMP)-2 and LMP-7. All of these genes are expressed at defined and distinct levels in normal tissues, and are inducible by IFN- γ . While the *cis* elements involved in transcription of the MHC class I heavy chain, β_2m , TAP-1 and LMP-2 have been analyzed extensively, those for TAP-2 and LMP-7 have not been well studied. Here we systematically analyzed the *cis* elements for TAP-2 transcription. We found at least two independent elements that are sufficient to activate transcription of a reporter gene. One (hereby called TAP-2 P1) is located 5' to the TAP-2 exon 1, while the other (hereby called TAP-2 P2) is a transcription initiator residing in intron 1. Analysis of the 5' sequence of TAP-2 mRNA indicates that both promoters are active. Moreover, while the TAP-2 promoter region contains *cis* elements that can mediate TAP-2 induction by IFN- γ , such as γ -activation site and IFN response factor binding element (IRFE), only the IRFE is required for IFN- γ induction of TAP-2 promoter *in vitro*. The IRFE appears to work as an enhancer for the initiator (P2). Together with another promoter recently identified by others, TAP-2 therefore has three independent promoters that can be differentially regulated.

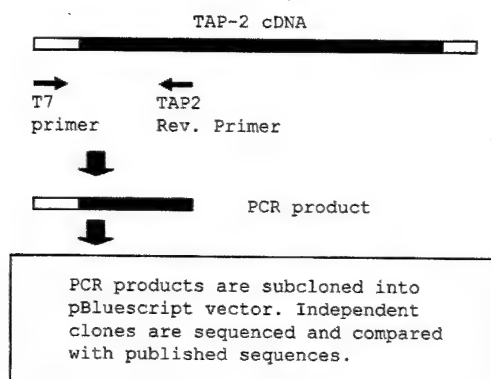
Introduction

MHC class I antigens are expressed constitutively in the majority of tissues, but the levels of expression differ significantly. While most leukocytes express high levels of MHC class I, other organs express these genes at much reduced levels, with almost no detectable expression in the central nervous system (1). In addition, MHC class I antigens are highly inducible by a number of cytokines, such as IFNs (2) and

tumor necrosis factors (3). Since MHC class I is the target for the majority of cytotoxic T cells (4), the expression level of these proteins determines the efficiency of immune surveillance by CD8 T cells. Indeed, both viruses (5,6) and tumors (7–11) can evade the immune system by down-regulating cell surface MHC class I expression.

Expression of MHC class I antigen on the cell surface

A Identification of TSS by PCR from two cDNA libraries



B TAP-2 cDNA 5' end PCR sequence

>Exon 1 (TSS):

Genomic DNA: CGCGGGACCC AGGCGCGCTC CCCGCGAGGG CGTCGCTGCG CACCCAGGAG ATCCAGTTTG AGAAGAAGCA GATTCCAGAA
 TAP2-1: CGCGGGACCC AGGCGCGCTC CCCGCGAGGG CGTCGCTGCG CACCCAGGAG ATCCAGTTTG AGAAGAAGCA GATTCCAGAA

>Intron 1

Genomic DNA: GCTCTCCTGA GGTGCCGCTC CGCAGCCGCA Ggtgagttct//.....//cacagacctc atttctccct ttctttccgc
 TAP2-1: GCTCTCCTGA GGTGCCGCTC CGCAGCCGCA G-----

>Exon 2 +1

Genomic DNA: agAACCCACC ATGGCGCTGT CCTACCTGAG GCCCTGGGTC TCTCTGCTGC TGGCGGACAT GGCTTTACTT GGGTTGCTAC
 TAP2-1: --AACCCACC ATGGCGCTGT CCTACCTGAG GCCCTGGGTC TCTCTGCTGC TGGCGGACAT GGCTTTACTT GGGTTGCTAC

TAP2 Rev. Primer

Fig. 1. Searching for TAP-2 exon 1 by RT-PCR. (a) Flow-chart of PCR and cloning of the TAP-2 5' fragment. Two pCDM8 (Invitrogen, Carlsbad, CA)-based cDNA libraries, one from the murine splenocytes and the other from the murine leukemia cell line RAW8.1, were used as templates. The T7.F forward primer and the TAP2.Rev reverse primer, which was based on the sequence of TAP-2 cDNA 45–70 bp down-stream of the translation initiation site, were used to amplify TAP-2 cDNA. The PCR products were cloned into the pBluescript vector. (b). Sequence of TAP-2 cDNA and alignment with genomic DNA. The inserts of three clones were sequenced and found to be identical. The sequence is also confirmed by bulk sequence of the PCR products. The first 111 bp were separated from the remaining cDNA fragment by a 663-bp fragment. The DNA encoding the 111 bp is hereby assigned as exon 1.

assay kit from Promega and is expressed as fold induction, calculated according to the following formula: fold induction = (sample luciferase/sample renilla luciferase)/(basic luciferase/basic renilla luciferase).

Data presented as means of triplicates with variations among replicates always <20%.

Characterization of 5' sequence of the TAP-2 cDNA by PCR
 cDNA libraries prepared from either RAW8.1 leukemia cell line or primary splenocytes were used as a source of TAP-2 cDNAs (19). The T7 primer was used as a forward primer and the reverse sequence corresponding to the 45–70 bp down-stream of the translation initiation codon of the TAP-2 gene was used as the reverse primer (TAP2.Rev). The PCR products obtained were either sequenced directly or were cloned into the pBluescript vector prior to sequencing.

Characterization of the 5' TAP-2 mRNA sequence by 5' rapid amplification of cDNA ends (5' RACE)

Total cellular RNA was isolated from splenocytes of either wild-type or STAT-1(–/–) C57BL/6j mice with Trizol reagent

(Life Technologies, Grand Island, NY). (5' RACE) was carried out with the 5' RACE system (Life Technologies). Briefly, 2 µg of total RNA was used for first-strand cDNA synthesis using random hexamer primers. The oligo(dC) tailed cDNA was amplified by PCR using Pfu DNA polymerase according to the protocol from the manufacturer (Promega; 35 cycles of 95°C for 1 min, 55°C for 0.5 min, 72°C for 3 min and final extension at 72°C for 5 min) with an abridged anchor primer (AAP, 5' RACE system; Life Technologies) and a TAP-2-specific primer (TAP2P1) complementary to nucleotides 454–478 of the TAP-2 coding sequence (5'-CCACAAGGAA-GAAGAAGGCAGCTAT) (GenBank M90459). A dilution of the original PCR product served as template for nested PCR using the abridged universal amplification primer (AUAP, 5' RACE system) and a second TAP-2-specific primer (TAP2P2) complementary to nucleotides 422–436 of the TAP-2 coding sequence (5'-GGCAGGTCCGGCCTGGACAGCTTCA). PCR products were separated by agarose gel electrophoresis, transferred to nylon membranes and hybridized to a TAP-2 cDNA probe. At the same time, larger DNA fragments

A DNA sequence of pTAP-2-P1F1:

GAGT CATTCTCTCT TGTAGTGGCC TGGTGGTGGG GTGTGGTGTAT GAGCAGCATG GTTGATTTC ATGTCTGAGA ATGGAACCTG GTGCCTCACC ATGCTCTGTG AGGGCTCTTC
 -1660 CCTGGAGGCC AGAAGGTGA TTTCCGCAAT GAAGTGGGCT CTGGGGAGGA GGCCGGGTTC CCTCTTTGAT GGTGTAGCC TACAGCCTAC TCTGACTACC TCACAGCAG CAGGCCCTGG
 -1540 CACTGACCCA GGGCCCTCAC TGCAAGTGGC GTGTGCTGCG AAGAAAGTCT GAGGACAAAG GGCCAGAGCC TCTGGGTCA AAATCTGTGA AAGGGAACCT TTGGCCTGCT GATAGGAGCT
 -1420 GAGGCCCTCT TGTAGCCCT AGTTTATCCC CAGACCCCAA CTCTTGAGCT TACCTGGAAA AACAAACAAG CCCTCAAGCC TCTCTTCTCT TCACCCCTCC GTCTTCTCT CTCTCTCTT
 -1300 CCCTTTTCCA AAGGTGGGAC ACCTGTGTGG TGTGCTGACT GGGAGGGACC TGAAGTCTCT CCCTCTCTCA TTTCTCTCCC TCCCTTCTCT TCCGTCACCG ACCATTCTCT TCCTCGCCT
 -1180 TTCTCTCTCT CCTATGCTCT TTTCTGCCCT TTAATCTCTC TCCTCTTTCC CTCTCTCTCA CCTACTGGC AGCGAACCTT GGGCTCAGGC AAGTTTCTC AACATCTGAA CTATAGTCCC
 -1060 AGTTTAACCG GCGCTTCTTA AAACATGCTC GGAGGTCGGA GAAATGCAGG GTTTTTTCGT TTTTTTTTTT TGTTTGTGTT GTTTTTTTTT GTTTTGTGTT TTGTTTTTTT TTCTTTTCA
 -940 AAGCATCTCA GACCTCAAG AGTTTGTCTA GTAGCGGCTT TGGAGGCGCA GCCTTTTTCG GAACCTGGGG CGCTCAACCC ACCCGCAAT TGACAGGCGC CATCTGCTGG CGGCTGTGCG
 -820 CAGCACCGCG ACCACGGCAG TCAAGTGAAA GCGAAAGCG CGGGACCCAG CGCGCTCCC CGGAGGGCG TCGCTGCGCA CCCAGGAGAT CCAGTTTGAG AAGAAGCAGA TTCCAGAAGC
 -700 TCCTCTGAGC TGCCGCTCCG CAGCCGCAAG TGAGTCTTT TGATAGAAC CCGAGAGTTA GCAGGAGTGC GGCCCATTC CAAACCCCTG GGTCAATCAT CCGGTTCGCC ACTCTCGTC
 -580 CCCTGTCTTG GTCTTTCTGC CGCTTACTCC CTGCCCCGTG TCCTCTGCTC CGTGCCCTTG CCACCCAGC TGTCCAGGT CCCCTGTCCC TCACCCCTTG CCCAAGGTCC TCIGCCCCAC
 -460 CCCTTGCCC TGCGTCCCGG GTCTTCCGTC GGGCGCGCCG GGCCACGCG CCTACTCAG AACCTTCTGT CCCAAGCAT CTAAGGCTAC CGAACCCACC CGATCCCGC CGCTGCACCG
 -340 GGTCTCTGGS CTCTTAAAGA CCCGAGGAAT GGGGCGAGTG TGTCTTCTTG GGTACACACC AACAAATTC AGGGAGATGG ATGTGTTGGC AGCAGACCGC CCCTCTCCCC ACCCCACCCG
 -220 CCGTGTCTG TGCTGTAGTG TGTGTGTGTC TGTGTGACTG TGTGTGAGAA TAATGCAGCG TTCCAGCTC CGTCAACAGC TCAGTCTAG GAGAGACTTA TCTAGGAGGG GGTCTTTCCG
 -100 GTGAACAAGA AGCCTCAGGG TGGATTGGG GCAGGAGGCT CGTAACCAGG GTTGAAGTC CACAGACCTC ATTCTCCCT TTCTTTCCGC AGAACCACCC ATG
 +1

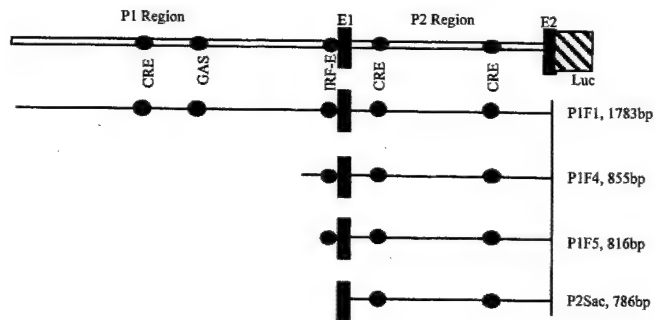
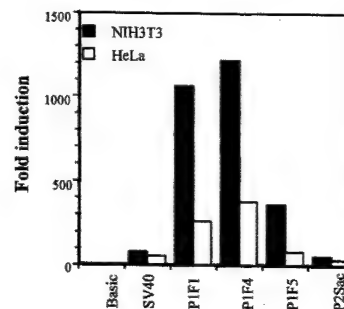
B pTAP-2 Promoter: P1 and P2 regions**C** Promoter activity

Fig. 3. Identification of the first promoter sequence 5' of exon 1. (a) Known *cis* elements in the 1.7-kb DNA fragment 5' to TAP-2 (P1F1). Numbering of the sequence is relative to the ATG codon of TAP-2 (thick bent arrow). The sequences of potential *cis* elements are boxed, the sequences of exon 1 and part of exon 2 are underlined, and three transcription starting sites (TSS) are indicated by bent arrows. (b) Diagram of the deletion mutants. (c) Deletion analysis revealed the critical function of a 70-bp fragment, 5' of exon 1, for the promoter activity of the 1.7-kb fragment. Constructs with deletions upstream of exon 1 were transfected to either NIH3T3 or HeLa cells and the promoter activity was determined by the dual-luciferase assay.

X75306 and X75307). The high GC contents in exon 1 (76/111) may explain the difficulties we encountered in primer extension and RNase protection assay.

The DNA sequence 5' of the TAP-2 open reading frame was not published when our analysis was initiated. Since the LMP-7 and TAP-2 genes are closely linked within the MHC class II region (Fig. 2a), we decided to isolate the intervening sequence using a pair of PCR primers based on available murine LMP-7 and TAP-2 sequences. The forward primer consisted of the 3' UTR of LMP-7 (LMP7.F1), while the reverse primer sequence corresponded to the 5' coding exon of TAP-2 (TAP2.R1). Using these primers, a 3.8-kb PCR fragment was amplified from 129/sv mouse spleen DNA and cloned into pBS-SK vector. Partial DNA sequence of ~600 bp from either terminus revealed that the 5' sequence of the 3.8-kb fragment was identical to the LMP-7 3' UTR, while the 3'

sequence was identical to the 5' portion of TAP-2. While this work was in progress, 138 kb of sequence from the murine MHC class II region was submitted to GenBank by Hood and colleagues (AF027865). The partial sequence we obtained was identical to the region between LMP-7 and TAP-2. Further restriction mapping indicated that the DNA fragment we had cloned was identical to the published sequence (data not shown).

As a first step towards characterizing the promoter region that controls TAP-2 expression, we compared the promoter activity of a 1.7-kb 3' fragment (P1F1) with that of the full-length 3.8-kb fragment (P1P2). The two fragments were cloned into the pGL2 luciferase reporter vector and transiently transfected into NIH3T3 or embryonic fibroblast cell lines prepared from either wild-type or STAT-1(-/-) C57BL/6j mice. After 48 h, the cell lysates were analyzed by dual-luciferase

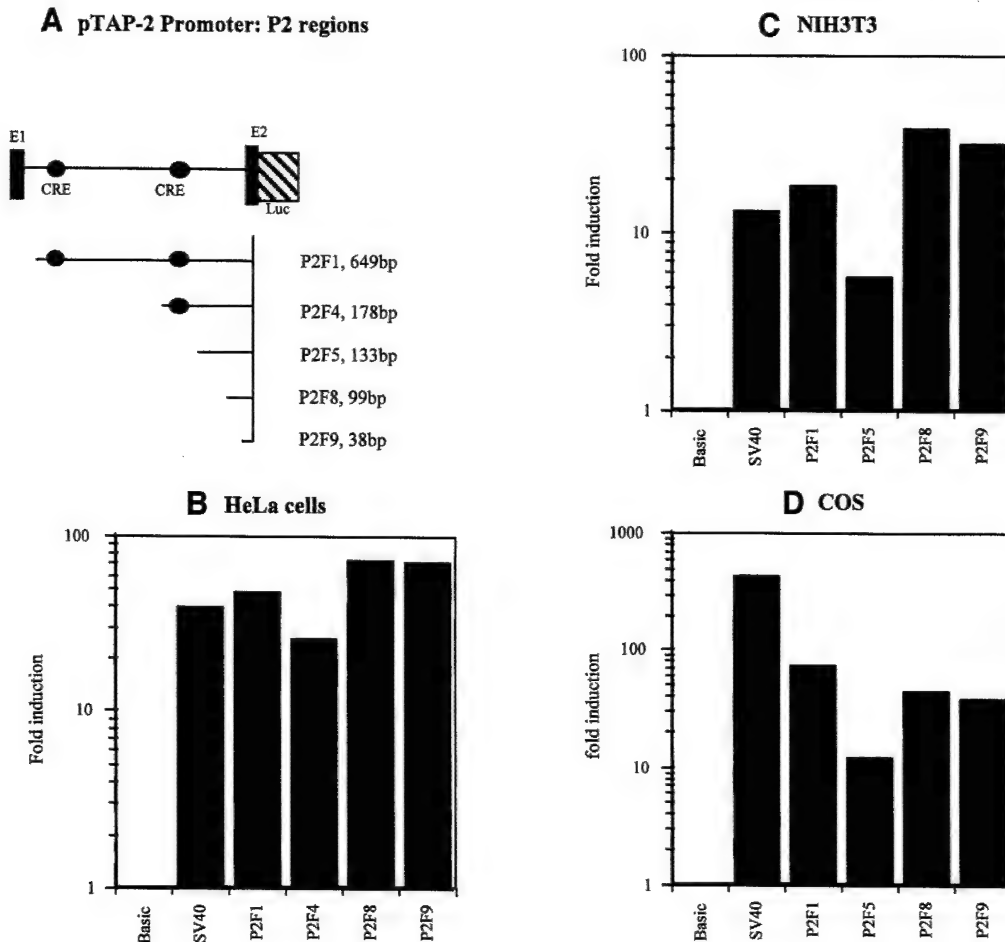


Fig. 5. Characterization of TAP-2 promoter 2. (a) Diagram of the *cis* elements in P2 region and the constructs used. (b–d) The constructs were transfected into HeLa (b), NIH3T3 (c) and COS (d) cells to test their promoter activity 48 h after transfection.

which started precisely at the +1 site of the *Inr* (Fig. 6a). This precise initiation strongly suggested that they are the products of transcription using *Inr* as the promoter. Group 3 clones (TAP-2-4) have a 5' terminus 9 bp down-stream of the putative translation initiation ATG codon. Because a second in-frame ATG start codon was present at position 49 after the first, it is possible that TAP-2-4 mRNA can still encode a truncated TAP-2 protein. Nevertheless, it is unclear whether this last group of cDNA reflects real mRNA product *in vivo* and, if so, whether this truncated TAP-2 protein can be functional.

In order to measure the relative abundance of the three groups of the TAP-2 cDNA, the 5' RACE products were separated by agarose gel electrophoresis and then analyzed by Southern blot using TAP-2 cDNA as the probe. Inserts from all three groups of cDNA clones were isolated and used as markers. As shown in Fig. 6(c), four major species of the 5' RACE products were detected by Southern blot. The molecular weights of these bands were consistent with the four transcription start sites we have predicted. It is therefore likely that the 5' termini of the major TAP-2 mRNA species have now been identified. Moreover, since all species were present in the RACE products from both wild-type and STAT-

1(–/–) spleens, both promoters must have been functionally independent of STAT-1.

*IRFE as an essential IFN- γ responsive element that acts as an enhancer for *Inr**

An important feature of genes involved in antigen presentation is their induction by IFN, especially IFN- γ . The TAP-2 promoter region contains a GAS and an IRFE. To determine whether any of these *cis* elements are required for IFN- γ -mediated induction of TAP-2, we carried out a systematic deletion analysis. The deletion mutants were cloned into the luciferase reporter constructs, as illustrated in Fig. 7(a), and then used to transfect either wild-type or STAT-1(–/–) embryonic fibroblasts. Transfected cells were left untreated or treated with 1000 U/ml of IFN- γ and lysates were tested for luciferase activity at 48 h. The ratios of luciferase activity in IFN-treated over untreated cultures are presented in Fig. 7(b). The data showed that deletion of all but 32 bp 5' of exon 1 had no effect on IFN- γ -induced TAP-2 promoter activity. Since the deletion of GAS had no effect on IFN- γ responsiveness, the GAS was not necessary for induction by IFN- γ . In contrast, IFN- γ induction was eliminated after a 32-bp sequence was

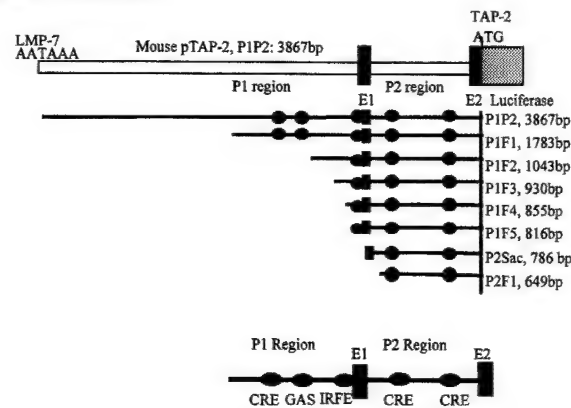
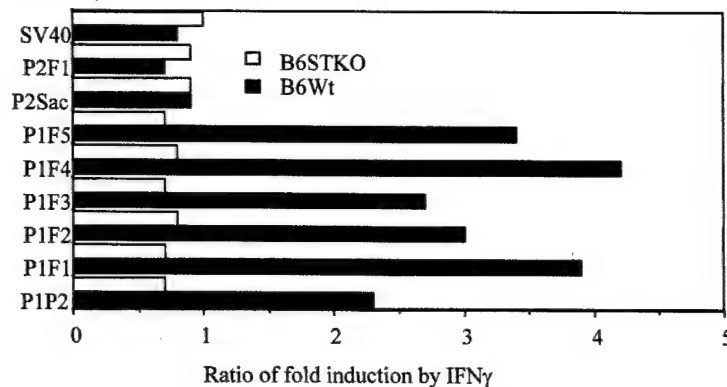
A Diagram of constructs**B IFN γ induction**

Fig. 7. IRFE, but not a GAS element, is involved in IFN- γ -mediated induction of the TAP-2 promoter activity. (a) Diagram of the *cis* elements in P1 and P2 regions, and the constructs used. (b) The constructs were transfected into the embryonic fibroblast cell lines prepared from either wild-type (B6WT, filled bars) or STAT1(-/-) (B6STKO, open bars) C57BL/6j mice. After transfection, the cells were incubated with medium or medium containing 1000 U/ml of IFN- γ for 48 h and the promoter activity was determined. Data presented are the ratio of fold induction of promoter activity from IFN- γ -treated culture over that from the untreated culture.

of antigen presentation, very little information is available on the gene structure and regulation of TAP-2 transcription. Our results presented here, and a recent publication by Arons *et al.* (21), provide the much-needed initial characterization. Taken together, the two studies reveal three distinct promoters responsible for expression of TAP-2 mRNA, two of which are responsive to the IRFE-mediated induction by IFN- γ .

The first promoter, P1, resides immediately upstream of exon 1. Deletion analysis revealed that essentially all of the promoter activity is contained in a 70-bp fragment 5' of exon 1. A lower, but significant promoter activity can be detected in an even smaller 32-bp fragment containing the IRFE sequence. Interestingly, while these short fragments have promoter activity, they contain neither a TATA box nor an Sp1 consensus sequence. The high G/C content of this region may allow binding of RNA polymerase to initiate transcription. The existence of this promoter is supported by Arons *et al.* (21), who reported a significant promoter activity of a 95-bp fragment encompassing both the 32- and 70-bp fragments. The second promoter, P2, resides 38 bp 5' of the translation start codon. Sequence analysis of this region indicates that

a transcription initiator (Inr) is located within the region. A signature of Inr function is that transcription starts at the +1 position of the Inr (22,23). Analysis of the 5' sequence of TAP-2 transcripts identified using 5' RACE indicates that the Inr is indeed employed as a second promoter. However, Arons *et al.* (21) were not able to observe any TAP-2 transcripts initiated in the intron 1 by RNase protection assay. This is most likely due to technical difficulties associated with the high G/C content of this region, as the majority of the 5' initiation sites were not identified by this method (21). The third promoter, identified by Arons *et al.* (21) (hereby called TAP-2 P3), encompasses 111 bp of exon 1. This region contains two multiple starting site down-stream element (MED1) sequences which may explain the multiple initiating sites identified by Arons *et al.* (21). It is unclear whether the difference in our results is due to technical difficulties or reflects the use of different cell lines in our respective studies.

It is worth noting that while all three promoters can function independently, in a physiological context they are most likely to function in concert. This is underscored by the requirement of the first intron for optimal functioning of the first promoter,

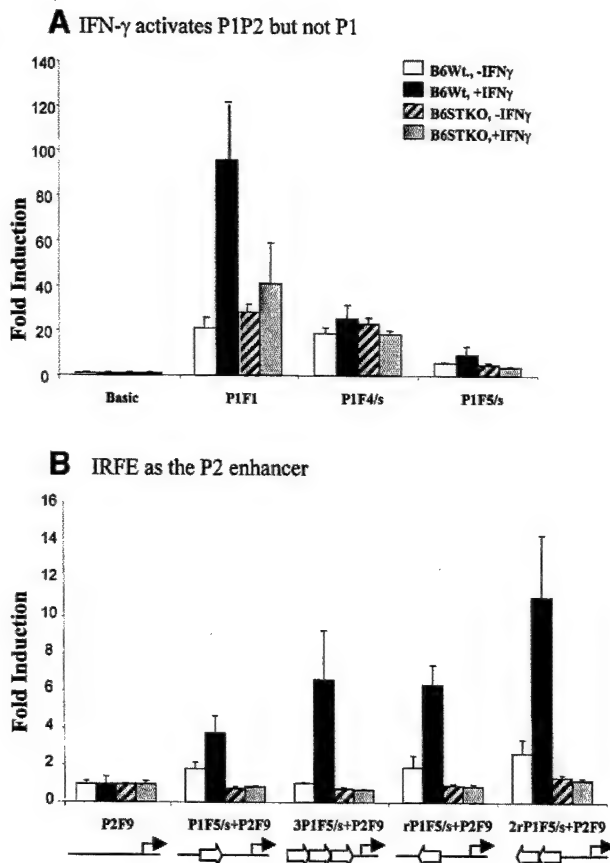


Fig. 9. The IRFE is an IFN- γ -activated enhancer for the transcriptional initiator. (a) Promoter 1 is not responsive to IFN- γ stimulation. The constructs P1F1, P1F4/s and P1F5/s (as in Fig. 4a) were transfected into the embryonic fibroblast cell lines prepared from either wild-type (B6WT) or STAT-1(-/-) (B6STKO) C57BL/6j mice. After transfection, the cells were incubated with medium (-IFN- γ) or medium containing 1000 U/ml of IFN- γ (+IFN- γ) for 48 h and the promoter activity was determined. Data shown were means \pm SD of luciferase activity expressed as fold induction. (b) The IRFE enhances the initiator activity upon IFN- γ stimulation. The 32 bp P1F5/s, which contains the IRFE, was inserted 5' of the P2F9, which contains the Inr, in different copy numbers and orientation as indicated. The constructs were transfected and assayed as described in (a). The data were normalized over the activity of P2F9 to illustrate the function of IRFE. Data shown are representative of three to five independent experiments.

but not IRF-2 and IFN consensus sequence binding protein, is sufficient to induce the TAP-2 promoter. Second, Stat-1 is required for optimal expression of IRF-1 (18,30). In this regard, it is most likely that activation of TAP-2 promoter requires both Stat-1 and IRF-1, much as what has been suggested for LMP-2 (31). Recent studies revealed an alternative pathway in which IFN-activated Stat-1 complexed with IRF-9 (p48) can activate transcription through interaction with the IFN-stimulated response element (32-34). The function of IRF-9 in the binding of TAP-2 promoter is not clear, as our preliminary studies indicated that anti-p48 did not super-shift the IRFE-protein complex from IFN- γ -stimulated cells (data not shown).

In summary, our analysis and that of Arons *et al.* (21) indicate that, despite the presence of numerous *cis* elements,

relatively few are involved in both constitutive and IFN- γ inducible expression of the TAP-2 gene. The constitutive expression is controlled by three promoters, a 70-bp fragment 5' to the exon 1, the MED1 sequence within exon 1 and an initiator located 32 bp 5' of the translation start codon. Identification of these *cis* elements will facilitate characterization of the transactivating element that controls TAP-2 gene expression. Moreover, since the promoter activity dissected in our study is based on analysis of five different cell lines from three different species, murine, monkey and human, it is most likely that the elements identified may function in a number of contexts. The existence of distinct promoter and transcription initiation sites allows independent mechanisms for expression of TAP-2. This apparent redundancy will likely make it more difficult to inactivate transcription by virus and tumors, while providing ample opportunities for interplay of multiple pathophysiological factors.

Acknowledgements

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Abbreviations

5' RACE	rapid amplification of 5' cDNA end
CRE	cAMP response element
GAS	γ -activation site
IRF	interferon regulatory factor
IRFE	interferon response factor binding element
LMP	low-molecular-weight polypeptide
MED1	multiple start site element down-stream
TAP	transporter associated with antigen processing
UTR	untranslated region

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Clonal Deletion of Simian Virus 40 Large T Antigen-Specific T Cells in the Transgenic Adenocarcinoma of Mouse Prostate Mice: An Important Role for Clonal Deletion in Shaping the Repertoire of T Cells Specific for Antigens Overexpressed in Solid Tumors¹

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In addition to their overexpression in cancer cells, most of the tumor-associated Ags are expressed at low but detectable levels in normal tissues. It is not clear whether the repertoire of T cells specific for unmutated tumor Ags is shaped by negative selection during T cell development. The transgenic adenocarcinoma of mouse prostate (TRAMP) model is transgenic for the SV40 large T Ag (Tag) under the control of the rat probasin regulatory elements. Although it has been established that T lymphocytes from TRAMP mice are tolerant to SV40 Tag, the mechanism of the tolerance is largely unknown. To examine whether the T cell clonal deletion is responsible for the tolerance, we crossed the TRAMP mice with mice transgenic for a rearranged TCR specific for SV40 Tag presented by the H-2K^k. Double transgenic TRAMP/TCR mice showed profound thymic deletion of SV40 Tag-reactive T cells, including a 6- to 10-fold reduction in the total thymocyte numbers and a >50-fold reduction in phenotypically mature T cells. Consistent with this finding, we observed that the SV40 Tag and endogenous mouse probasin genes are expressed at low levels in the thymus. These results demonstrate that clonal deletion is a major mechanism for tolerance to Ags previously regarded as prostate-specific, and provide direct evidence that the T cell repertoire specific for an unmutated tumor Ag can be shaped by clonal deletion in the thymus. *The Journal of Immunology*, 2002, 169: 4761–4769.

The majority of the tumor Ags identified so far has the same sequences as the endogenous genes (1–7). These unmutated tumor Ags are often recognized by T cells from cancer patients (8–13). However, with the notable exception of the PR-1 Ag in chronic myelogenous leukemia (10), cancers appear to progress despite significantly expanded CD8 T cells specific for the tumor Ags. It has been suggested that these T cells are either anergic as a result of peripheral tolerance (9) or have low avidity for the cancer Ags (14). In the model of experimental autoimmune encephalomyelitis, it has been documented that T cells in mice lacking the autoantigen myelin basic protein have an increased avidity for this Ag (15). Similarly, T cells from mice with a targeted mutation of tumor suppressor gene *p53* (16) or spontaneous mutation to tyrosinase (17) have intrinsically higher affinities than T cells from the wild-type mice. A recent study demon-

strated that tolerance to SV40 large T Ag (Tag)³ resulted in unresponsiveness to the dominant, but not the subdominant, Tag epitope (18). Therefore, it is likely that tolerance to self Ags removes T cells with high avidity to the immunodominant epitopes on unmutated tumor Ags. However, it is unclear whether high avidity T cells are removed by thymic clonal deletion or by mechanisms of peripheral tolerance.

Sarma et al. (19) examined the negative selection of T cells specific for unmutated tumor Ags using mice transgenic for Ag-specific TCR. The results indicated that transgenic T cells specific for the unmutated tumor Ag P1A develop normally unless the tumor Ag is transgenically overexpressed in the thymus. However, since the transgenic TCR was isolated from a CTL clone that had gone through negative selection in mice that expressed the tumor Ag at low levels, it is not surprising that transgenic T cells escaped clonal deletion. Thus, this work did not address whether the repertoire of T cells specific for the unmutated tumor Ag is subjected to negative selection in the thymus. For this purpose one must start with TCR isolated from mice lacking specific Ag, and investigate the fate of T cells in mice that express this Ag.

In addition to being a major Ag, SV40 Tag is a potent oncogene. Tissue-specific expression of SV40 Tag leads to development of tissue-specific cancers, including cancers in liver (20), brain (21, 22), bone (23), and pancreas (24). Recently, Greenberg et al. (25) have described a transgenic mouse model for prostate cancer: transgenic adenocarcinoma of mouse prostate (TRAMP) mouse. In this model, a minimal rat probasin promoter regulatory element

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³ Abbreviations used in this paper: Tag, large T Ag; TRAMP, transgenic adenocarcinoma of mouse prostate; PAE, peripheral Ag-expressing; DC, dendritic cell; mPB, murine probasin.

effector cells and target cells were added to the 96-well plate at different E:T ratios. After 6 h, the released ^{51}Cr in the supernatants was harvested and determined by a Packard TopCount NXT Microplate Scintillation and Luminescence Counter (PerkinElmer, Downers Grove, IL). The specific percentage of lysis was calculated as: $\text{specific \% lysis} = [(\text{cpm}_{\text{samples}} - \text{cpm}_{\text{medium}}) / (\text{cpm}_{\text{maxim}} - \text{cpm}_{\text{medium}})] \times 100$. The data presented are means of duplicates.

Histology and immunohistochemistry study

Freshly harvested mouse thymus, spleen, and prostate tissues were snap frozen in isopentane and stored at -70°C . Five-micrometer thick sections were cut with a cryostat (Microm HM 505E; Fisher Scientific, Fairlawn, NJ) at -20°C , and frozen sections were fixed with acetone before staining with H&E. For double label immunostaining, we used VECTASTAIN Elite ABC (avidin/biotin complex) kits following the protocol provided by manufacturer (Vector Laboratories, Burlingame, CA). Briefly, the frozen sections were fixed in cold acetone and blocked with 1% H_2O_2 , avidin-blocking solution, and biotin blocking solution (Vector Laboratories), respectively. This was followed by preincubation with 5% normal horse serum in PBS for 30 min. The sections were then incubated with mAb to SV40 Tag (Pab 101; BD PharMingen) for 1 h. The biotinylated horse anti-mouse IgG was applied to the slides as a secondary Ab, followed by application of avidin-biotin-peroxidase complex (ABC; Vector Laboratories). The Vector VIP (dark purple; Vector Laboratories) was used as first step enzyme substrate. The sections were treated with 1 N HCl for 10 min and preincubated with 10% goat normal serum for 10 min. The sections were then incubated with hamster anti-mouse CD11c (HB224) supernatant for 1 h. The biotinylated goat anti-hamster IgG (Jackson ImmunoResearch Laboratories, West Grove, PA) was applied to the slides as a secondary Ab, followed by application of ABC reagents. Diaminobenzidine (Vector Laboratories) was used as a second step enzyme substrate. All sections were counterstained in Vector's Methyl Green Counterstain (Vector Laboratories) and then mounted with Permount histologic mounting medium (Fisher Scientific). Histological and immunostained slides were studied and photographed using an Olympus BX-40 microscope (Olympus, Melville, NY).

Results

Both male and female TRAMP mice are tolerant to an immunodominant CTL epitope in SV40 Tag

Male TRAMP mice developed immune tolerance against the SV40 Tag as they failed to respond when immunized with SV40 Tag-transfected fibroblast B6-3T3 cells (33). As the first step to determine whether prostate expression of Tag is responsible for the tolerance, 8- to 12-wk-old male or female C57BL/6 background TRAMP mice and their Tag-negative littermates were immunized s.c. with 100 μg of MHC H-2K^b-restricted immunodominant Tag epitope IV peptide (residue 404–411; Ref. 29) in CFA. After 9–10 days, the mice were sacrificed and draining lymph node cells and splenocytes were collected. The frequency of Tag epitope IV-specific IFN- γ -producing cells were determined by ELISPOT assay. An unrelated H-2K^b peptide from HSV-1 gB peptide was used as control (47). As shown in Fig. 1, Tag-negative male mice (Tag⁻ Imm) developed a vigorous response to immunodominant Tag epitope IV, while the male TRAMP mice produced a barely detectable T cell response, consistent with prior studies that revealed immune tolerance to this epitope in male TRAMP mice (33). Surprisingly, the number of Tag-specific T cells was also low in female TRAMP mice in comparison to Tag-negative littermates. Thus, TRAMP female mice are also tolerant to this Ag. As expected, the numbers of Tag-reactive cells were not significantly different between male and female mice. These results indicate that expression of Tag in the prostate is not required for induction of tolerance to Tag in TRAMP mice.

Thymic clonal deletion of transgenic T cells in TRAMP/TG-B double transgenic mice

TG-B mice expressed a rearranged TCR from a CD8⁺ cytotoxic T cell clone that recognized SV40 Tag presented by MHC class I molecule H-2K^k (44). The presence of transgenic T cells can be monitored by flow cytometry with anti-CD8 and anti-V β 8-specific

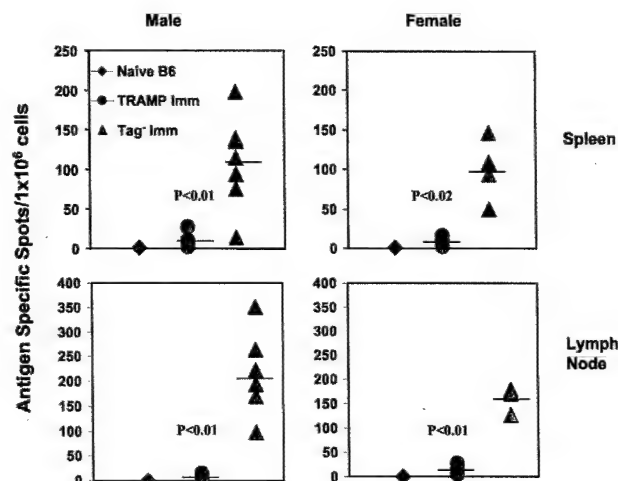


FIGURE 1. Both male and female mice are tolerant to SV40 Tag. Male and female TRAMP mice and their nontransgenic littermates (Tag⁻ Imm) were immunized with the major Tag epitope. At 9 days after immunization, the spleens and lymph nodes from naive and immunized mice were harvested and Tag-specific IFN- γ -producing T cells were measured by ELISPOT assay. Six-point titrations of cells were applied to the culture in 96-well plates containing either 1 $\mu\text{g}/\text{ml}$ Ag-specific peptide SV40 Tag epitope IV or 1 $\mu\text{g}/\text{ml}$ control peptide HSV gB epitope. The spots were counted under a contrast microscope. The SV40 Tag Ag-specific spots were calculated by subtracting the means of correlated nonspecific HSV gB peptide IFN- γ spots from the means of SV40 Tag epitope IV peptide IFN- γ spots. Linear regression analysis on the six-point titration was used to obtain the Ag-specific spots per 10^6 splenocytes or lymph node cells. Data shown are summaries of three independent experiments.

mAbs. To test whether the tolerance was caused by thymic clonal deletion of SV40 Tag-reactive T cells, we bred the TRAMP mice with TG-B mice to obtain TRAMP/TG-B double transgenic and TG-B single transgenic F₁ (H-2^{b^{bk}}) mice. The fates of SV40 Tag-specific T cells in the central and peripheral lymphoid organs were examined in the F₁ mice at 25–30 days after birth. Gross anatomic examination showed that the thymi of TRAMP/TG-B double transgenic mice were wrinkled and much smaller than those of nontransgenic or TG-B single transgenic mice. This was consistent with the number of viable cells recovered from the thymus (Fig. 2a). In male double transgenic mice, the total thymocytes were 1.1×10^7 cells in average compared with 6.5×10^7 cells in TG-B single transgenic F₁ mice. Thymocytes were then stained with anti-CD4, anti-CD8, and anti-V β 8 Abs and examined by three-color flow cytometry. The upper panels in Fig. 2b depict the composition of the thymocytes of the male mice, while the lower panels show those of the female mice. The percentages of CD4⁺CD8⁺ cells and CD8⁺CD4⁻ cells were significantly decreased in TRAMP/TG-B double transgenic mice in comparison to those in TG-B single transgenic mice. The female double transgenic mice also showed a profound decrease in these two populations as the male mice did. Among the V β 8^{high} cells, CD8⁺CD4⁻ cells were reduced by >50-fold in the thymus of TRAMP/TG-B double transgenic mice (Fig. 3a). The majority (74%) of V β 8-positive cells were CD4⁻CD8⁻ (Fig. 3b, upper right), which is consistent with clonal deletion occurring at early CD4⁺CD8⁺ stage of development as has been seen in other MHC class I-restricted TCR-transgenic mice (36, 37). Therefore, these data demonstrate that the SV40 Tag-specific transgenic T cells are efficiently deleted in the TRAMP/TG-B double transgenic mice. The clonal deletion in the female double transgenic mice was at least as complete as in the male mice, as suggested by severe reductions in the proportion of CD4⁺CD8⁺ and CD8⁺CD4⁻V β 8^{high} T cells in thymus

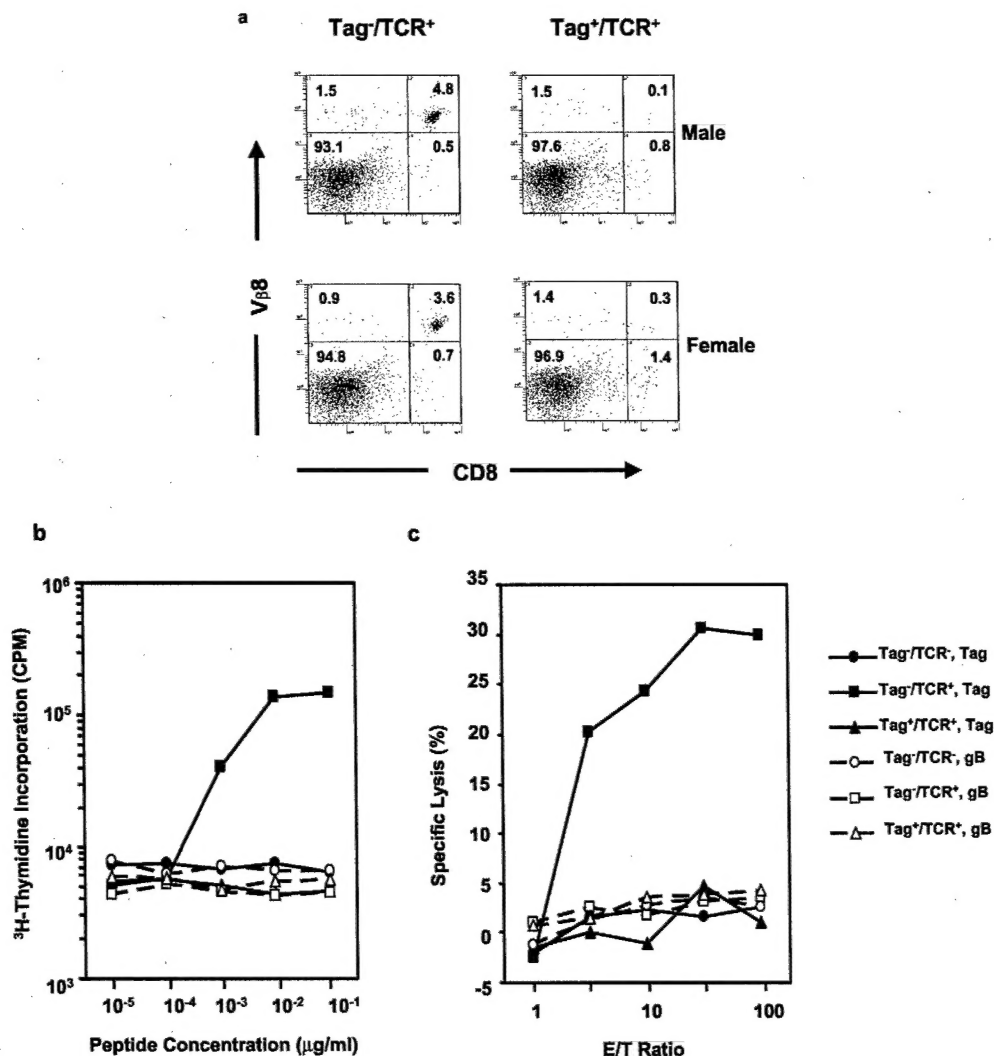


FIGURE 4. Numbers and functions of mature Tag-specific T cells in the spleen. Spleens from SV40 Tag⁺/TCR⁺ double transgenic F₁ mice and SV40 Tag⁻/TCR⁺ single transgenic F₁ mice were harvested at day 25 after birth. *a*, Splenocytes were stained with anti-CD4, anti-CD8, and anti-Vβ8 Abs and examined by three-color flow cytometry to detect the percentage of the Vβ8⁺CD8⁺ transgenic T cells. The numbers in the quadrants were percentages of cells. *b*, Proliferation of Tag-specific T cells. Splenocytes were stimulated for 66 h in the presence of Tag peptides 560–568 (solid lines) or control HSV gB peptides (dashed lines) in different concentrations as indicated, and proliferation was detected by pulsing the culture with [³H]Tdr for an additional 6 h. *c*, The cytotoxicity of Tag-specific T cells. Splenocytes were cultured with 0.1 μg/ml Tag peptide 560–568 for 5 days. Viable cells were isolated from the interface between Ficoll-opaque solution and the medium and were used as effectors. Tag-peptide pulsed (solid lines) or HSV gB peptide (dashed lines) pulsed ⁵¹Cr-labeled L929 cells were used as target cells. Data shown are from one representative of five independent experiments.

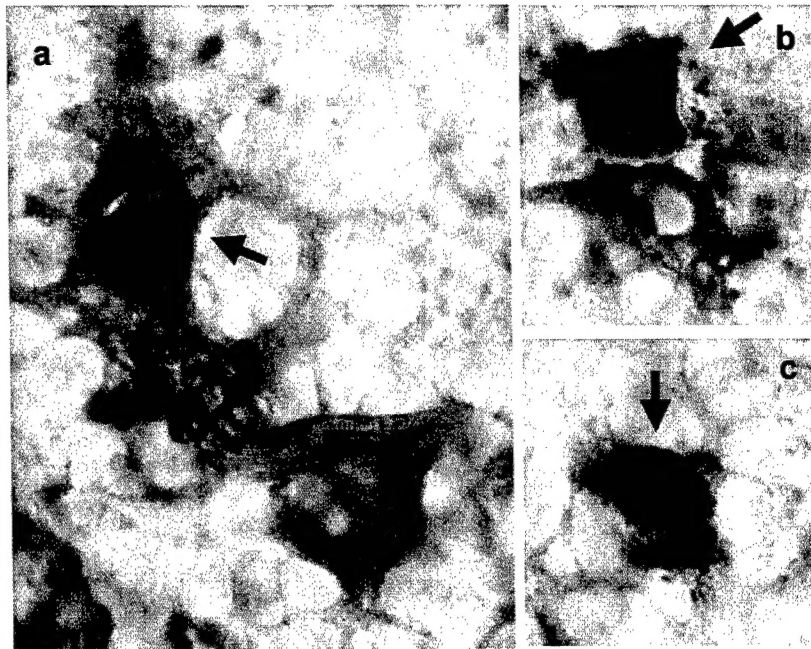
of Tag expression in the thymus. Contamination of DNA was excluded by two criteria: 1) the size of products were as predicted after RNA splicing (260 bp); and 2) no product of this size was detected unless the reverse transcriptase was used. The PCR products were sequenced to confirm the correct splicing form of Tag. As shown in Fig. 5*a*, by using this detection method, we were able to reproducibly detect SV40 Tag expression in the thymus as early as 2 days after birth. Before puberty, we found that the SV40 Tag expression level was higher in the thymus than that in the prostate gland (Fig. 5*b*). The SV40 Tag expression increased >1000-fold in the prostate gland after puberty, which was consistent with the androgen-mediated activation of probasin promoter (49). Interestingly, the thymic expression of SV40 Tag was not augmented in this process (Fig. 5*c*). Thus, thymic and prostatic Tag expressions were differentially regulated. As such, different molecular mechanisms may be responsible for thymic expression of the Tag gene.

The low levels of Tag expression in the thymus can be due to either poor expression on a large number of cells, or high expression on a small number of specialized cells. To address this issue, frozen

sections of TRAMP mouse thymus were immunostained with anti-Tag Ab Pab101 that reacted with the Tag located on nucleus and anti-CD11c Ab that stained the cell membrane. Examples of Tag-expressing cells were shown in Fig. 6. Some cells that express SV40 Tag also express CD11c, a marker for dendritic cells (DCs) (Fig. 6*a*, arrow). However, >95% of the DCs did not express Tag. A higher number of Tag expressing cells were not stained with anti-CD11c (Fig. 6, *b* and *c*), which indicates other cell types also expressed Tag. Moreover, while the majority of the Tag⁺ cells were located in the medulla, some were scattered in the cortical-medullary junction region and cortex (data not shown). The presence of the Tag⁺ cells at multiple sites might explain the strong clonal deletion of Tag-specific T cells starting at the CD4⁺CD8⁺ stage.

To rule out the possibility that expression of Tag is an artifact of the transgene, we also tested if the endogenous probasin is expressed in the thymus. mPB has been cloned and its expression has been shown to be prostate-specific (48). Using the same RT-PCR plus Southern blot method, we found that mPB mRNA was detectable in the thymus of TRAMP mice before puberty (25 days

FIGURE 6. Detection of Tag expression in DCs and non-DCs in the thymic medulla. The frozen sections of TRAMP mouse thymic tissue were double immunostained with anti-SV40 Tag Ab (Pab101; Vector VIP as substrate) and anti-CD11c (diaminobenzidine as substrate). The slides were photographed with an Olympus BX-40 microscope under $\times 100$ objective. Three representative Tag⁺ cells are shown. *a*, A CD11c⁺Tag⁺ cell (arrow) together with a CD11c⁺Tag⁻ cell. *b* and *c*, Two examples of Tag-expressing cells (arrows) that are not expressing CD11c marker. All examples are taken from thymic medulla, although both CD11c⁺Tag⁺ and CD11c⁻Tag⁺ cells can also be found at cortex and cortex-medulla junction areas (data not shown).



to observe consistent tolerance was attributed to low number of PAE cells (estimated to be within 100–300/thymus or ~ 1 cell/thymic section). To our knowledge, our study is the first example using TCR transgenic model to show complete clonal deletion in the thymus that may be attributable to expression of Ag in PAE cells.

It has been recently reported that thymic stromal cells, especially medullary epithelial cells, promiscuously express various tissue-specific genes (57, 62, 64, 65). However, other investigators support the view that bone marrow-derived APCs, such as DCs and macrophages, may play a key role in inducing immunologic tolerance and expressing self Ags (55, 58, 66). Our findings on double-immunohistochemical staining on Tag Ag and CD11c appear to indicate that the two schools of thought are not mutually exclusive. There is evidence that both bone marrow-derived APCs and thymic epithelial cells express self Ags with tissue-restricted expression in the thymus, although it is not clear whether their roles in central and/or peripheral tolerance are complementary or redundant.

In several Tag-induced spontaneous tumor models, including cancers developed in the liver (30), brain (31, 67), bone (32), and prostate (33), it has been reported that active immunizations failed to induce protection. Correspondingly, T cell response to immu-

nization by various forms of Tag was either absent or of extremely low avidity (30–33). Because transfer of either naive or activated T cells from Tag-negative mice provided partial or full protection, T cells in these various Tag-transgenic mice must be rendered tolerant to Tag. In the case of Tag-induced pancreatic cancer, the tolerance of T cells (35), and perhaps as result of which, the effect of active immunization was determined by the timing of Tag expression (34). Theoretically, immune tolerance can be achieved centrally in the thymus, or in the periphery, by either clonal deletion or by functional inactivation. The identification of thymic expression of the Tag in some transgenic mice has provided strong argument for a role of central tolerance (34, 53). However, due to the low frequency of Ag-reactive T cells in normal mice, previous studies have not clearly established whether the transgenic Tag can induce central or peripheral tolerance and whether clonal deletion or functional inactivation is responsible. The class I-restricted SV40 Tag-specific TCR transgenic mice, developed by Geiger et al. (44), provided a valuable tool to address this issue. However, these mice had been used mainly to reveal nondeletional mechanisms (44, 54, 68). In this study, we presented clear-cut evidence that clonal deletion can be induced by the oncogene previously thought to express in tissue-specific fashion. It is well-documented that Ags that are ubiquitously expressed, such as H-2 (37) and H-Y Ag (36, 69), can induce clonal deletion of MHC class I-restricted T cells. Our work extended these findings into peripheral Ags that can be expressed by the so-called PAE cells in the thymus.

Because unmutated tumor Ags are present in a high proportion of cancers of the same histological origin as well as those that are from different lineages (1–7), these Ags are the primary targets for immunotherapy. The nature of T cell repertoire to unmutated Ags is of both fundamental and practical significance. Using mice transgenic for a TCR specific for unmutated tumor Ag P1A, Sarma et al. (19) reported that P1A-specific T cells are not deleted in wild-type mice with a low expression of P1A. At face value, this appears contradictory to the finding with Tag-specific T cells, as reported in this study. However, it is important to bear in mind that P1A-specific TCR was isolated in a CTL clone from mice that express the P1A gene (1, 19, 70). The very fact that these T cells can be produced indicates that this TCR is not deleted in wild-type mice. As such, the previous work did not address whether the

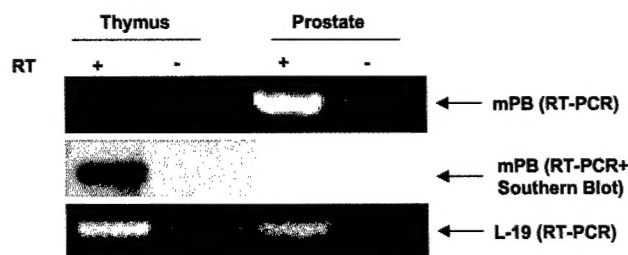


FIGURE 7. The thymic expression of murine probasin. RT-PCR followed by Southern blot was conducted in the RNA sample isolated from a 25-day-old TRAMP mouse. With exceptions of the primer and probe used, the experiments were performed as described in Fig. 5. The control house-keeping gene L-19 expression and murine probasin expression in prostate were shown as agarose gel images, while the murine probasin expression in thymus was shown as PCR-Southern blot ECL film image.

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Appendix 5.

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DISTINCT THYMIC CELL TYPES EXPRESSING PERIPHERAL TUMOR ANTIGEN
DETERMINE THE RANGE AND DEGREE OF CLONAL DELETION OF T CELLS

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Recent studies have shown that many peripheral antigens are expressed in specialized cells in the thymus and can induce central tolerance. It has been suggested that thymic medullary epithelial cells are responsible in tolerizing developing thymocytes, while some studies pointed to the bone marrow (BM) derived dendritic cells (DC) and macrophages. We have recently taken a double-transgenic approach to show that clonal deletion plays an important role in immune tolerance to tumor antigen SV40 large T antigen (Tag) in prostate cancer mouse model TRAMP mice. Our preliminary data suggested that both BM derived APC and thymic epithelial cells express Tag in the thymus. Here we carried out a BM radiation chimera mice study to determine the contribution of BM derived APC vs. thymic epithelial cells in clonal deletion process. We followed the fate of Tag specific transgenic CD8⁺V β 8⁺ T cells in thymus and spleen from different BM chimera mice that have distinct cell type expressing Tag. The results showed that clonal deletion of Tag reactive transgenic T cells is induced in the mice that Tag is expressed by thymic epithelial cells alone. The clonal deletion is aggravated in mice with both thymic epithelial cells and BM derived APC expressing Tag. An interesting observation is that the Tag specific T cell numbers, as well as antigen specific T cell function, are significantly decreased in the spleen from the mice that Tag is expressed in BM derived APC alone, although such T cell numbers in the thymus are similar to that of the control mice without clonal deletion. Our results suggested that distinct thymic cell types expressing peripheral tumor antigen have different roles in determining the range and degree of central and peripheral tolerance.